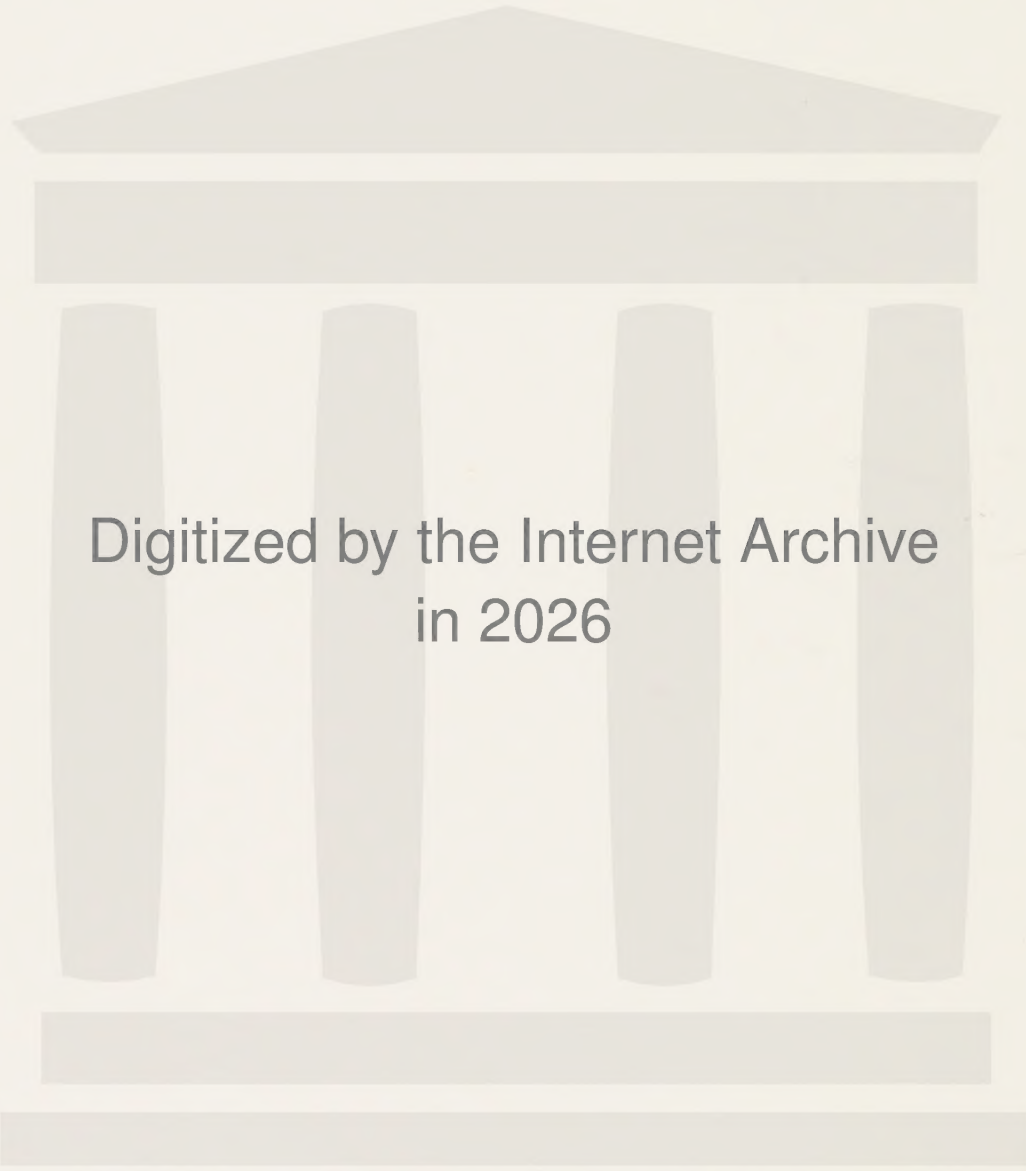


NMR STUDIES OF QUADRUPOLEAR IONS
IN AQUEOUS SOLUTION
AND BIOLOGICAL SYSTEMS

Pétur Reimarsson

Physical Chemistry 2
Chemical Center, P.O.B. 740
S-220 07 LUND, Sweden

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NMR STUDIES OF QUADRUPOLE
IN AQUEOUS SOLUTION
AND BIOLOGICAL SYSTEMS

John F. Meier



Physical Chemistry
Chemical Center, P.O. Box
30-3005 Leiden, The Netherlands

University of Lund, Dept. of Physical Chemistry 2.
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Pétur Reimarsson

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NMR Studies of Quadrupolar Ions in Aqueous Solution
and Biological Systems.

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^{35}Cl NMR (Nuclear Magnetic Resonance) studies of the interaction between perchlorate and different cations were performed. The results were discussed in terms of the electrostatic model for quadrupole relaxation. With Mn^{2+} as the cation the relaxation is dominated by paramagnetic effects. The possibility of using rather large symmetrical anions (ClO_4^- and AsF_6^-) to probe protein anion binding sites was discussed. $^{35}\text{Cl}^-$ NMR was used to probe anion binding to lactate dehydrogenase, malate dehydrogenase, alkaline phosphatase and halophilic ferredoxin. $^{23}\text{Na}^+$ NMR was used to probe cation binding to parvalbumin. $^{23}\text{Na}^+$, $^{25}\text{Mg}^{2+}$ and $^{43}\text{Ca}^{2+}$ NMR were used to probe cation binding to DNA. The interaction of polyamines and DNA was discussed as well as ion binding properties of chromatin.

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Pris

"He said science was going to discover the basic secret of life some day," the bartender put in. He scratched his head and frowned. "Didn't I read in the paper the other day where they'd finally found out what it was?"

"I missed that," I murmured.

"I saw that," said Sandra. "About two days ago."

"That's right," said the bartender.

"What is the secret of life?" I asked.

"I forget," said Sandra.

"Protein," the bartender declared. "They found out something about protein."

"Yeah, said Sandra, "that's it."

Kurt Vonnegut

(from "Cat's Cradle")

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BIOLOGICAL SYSTEMS

Pétur Reimarsson
Physical Chemistry 2
Chemical Center
P.O. Box 740
S-220 07 LUND, Sweden

The present thesis consists of this summary and the following papers:

- I. Quadrupole Relaxation of Chloride Ion, and of Perchlorate and Other Tetrahedral Ions in Aqueous Solution.
P. Reimarsson, H. Wennerström, S. Engström and B. Lindman, J. Phys. Chem. 81, 789 (1977).
- II. ^{35}Cl NMR Relaxation Study of the Interaction of Perchlorate With Metal Ions in Aqueous Solution.
P. Reimarsson and B. Lindman, Inorg. Nucl. Chem. Letters 13, 449 (1977).
- III. NMR Studies of the Interaction Between Mn^{2+} and ClO_4^- in Aqueous Solution.
P. Reimarsson, submitted for publication (1979).
- IV. The ClO_4^- Ion as a Probe in NMR Studies of Protein Anion Binding Sites.
P. Reimarsson, T.E. Bull and B. Lindman, FEBS Letters 59, 159 (1975).
- V. The AsF_6^- Ion as a Probe in NMR Studies of Protein Anion Binding Sites.
P. Reimarsson, T. Drakenberg and B. Lindman, J. Magn. Resonance 29, 169 (1978).
- VI. ^{35}Cl Nuclear Magnetic Relaxation Studies of Cl^- Binding to Lactate Dehydrogenase.
T.E. Bull, B. Lindman and P. Reimarsson, Arch. Biochem. Biophys. 176, 389 (1976).
- VII. NMR Studies of Chloride Ion Binding to Proteins.
P. Reimarsson, T.E. Bull, J.-E. Norne and B. Lindman in "Energetics and Structure of Halophilic Microorganisms",

p. 41, (S.R. Caplan and M. Ginzburg, eds.), Elsevier/
North Holland, Amsterdam (1978).

VIII. The Relation Between Activity and Zinc and Chloride
Binding of E. Coli Alkaline Phosphatase.

J.-E. Norne, H. Szajn, H. Csopak, P. Reimarsson and
B. Lindman, Arch. Biochem. Biophys., in press (1979).

IX. ^{35}Cl NMR Studies of Anion Binding to Ferredoxin from
Halobacterium of the Dead Sea.

P. Reimarsson, B. Lindman and M.M. Werber, submitted for
publication (1979).

X. Na^+ Binding to Parvalbumin Studied by ^{23}Na NMR.

J. Parello, P. Reimarsson, E. Thulin and B. Lindman,
FEBS Letters 100, 153 (1979).

XI. DNA as a Polyelectrolyte. Monovalent Cation Binding by
 ^{23}Na NMR.

D. Burton, S. Forsén and P. Reimarsson, submitted for
publication (1979).

XII. Counterion NMR in Polyelectrolyte Solutions. Preliminary
NMR Study of $^{25}\text{Mg}^{2+}$ and $^{43}\text{Ca}^{2+}$ Interaction with DNA.

P. Reimarsson, J. Parello, T. Drakenberg, H. Gustavsson
and B. Lindman, submitted for publication (1979).

XIII. ^{23}Na NMR as a Probe of Ion Binding to Chromatin.

D. Burton and P. Reimarsson, FEBS Letters 89, 183 (1978).

Schematically the subject of these papers can be divided into
three different topics.

- a) [I-III] concern the relaxation of the perchlorate anion in
aqueous solution. In [I] the relaxation in the limit of in-
finite dilution is discussed and interpreted in terms of
the electrostatic theory of ion relaxation. In [II] the
attention is focussed on the relaxation rates in the pre-
sence of different cations. For the case of Mn^{2+} as the
cation, the relaxation is mainly caused by the unpaired
electron spin of Mn^{2+} as discussed in [III].
- b) The interaction of ions with proteins. Thus in [IV] and [V]
the possibilities of using polyatomic anions of high symme-
try (ClO_4^- and AsF_6^- respectively) to study anion binding
sites are considered. In [VI, VIII and IX]

the anion binding properties of three different proteins are considered; the discussion in [VII] concerns mainly the internal motion of chloride bound to proteins. Some of the results on alkaline phosphatase in [VIII] are also discussed in [VII]. In [X] the sodium binding properties of parvalbumin are discussed.

- c) [XI-XIII]. In these papers, the cation binding properties of DNA are explored, with special attention to the polyelectrolyte behaviour of DNA. In [XIII] ion binding to chromatin is discussed.

2. INTRODUCTION

The NMR (nuclear magnetic resonance) spectrum of a quadrupolar ion in an isotropic solution consists of a single resonance line when conditions of fast exchange prevail. This line is generally described by a) the resonance frequency, b) the intensity, and c) the lineshape.

The discussion in this thesis is concerned with only one of these, namely, the lineshape which is in most cases given by two parameters. (The same information is obtained, if, instead of recording the spectrum, the NMR relaxation times (T_1 and T_2) of the ions are measured.) These parameters, and therefore the lineshape, are dependent on a number of variables, among which are ionic strength, temperature, counterions, solvent and the presence of macromolecules.

To construct a quantitative theory of the parameters determining the lineshape as a function of these variables is, if not impossible, very difficult, as a description of these systems on a molecular level is not available. It is convenient, in the present context, to distinguish between two particular cases:

- a) the study of solvated ions in solutions, and
- b) the study of ions in solutions containing macromolecules.

For the former a theoretical approach is available and at least a qualitative understanding of the relaxation of ions can be given.

In a solution containing macromolecules, the spectrum is affected by the bound ions and quantitative information can often be obtained on the four parameters which affect the lineshape i.e. the fraction of ions bound, the strength of interaction between ion and macromolecule, the motional characteristics of the bound ions and the rate by which the ions exchange between a site on the macromolecule and the bulk solution. These parameters in turn are dependent on the variables discussed above. It is thus clear that a great deal of valuable information can be obtained from NMR studies of ion binding to macromolecules. The research in this area has intensified in recent years, in the main because of the improved instrumental situation (the availability of Fourier transform techniques and the high field of superconducting magnets). Previously the

spectra were generally obtained by continuous-wave methods and it was difficult to obtain spectra at low ion concentrations. Now, however, NMR signals from 1 mM Cl^- and 0.1 mM Na^+ with good signal-to-noise ratio can be obtained in times of the order of minutes. These developments have facilitated the study of ions in biological systems, where low ionic concentrations are desirable.

The disposition of this summary is as follows. After discussion of some theoretical aspects, the relaxation of the perchlorate anion will be considered. There then follows a general discussion on the role of ions in biological systems whereupon the binding of ions to proteins is considered in more detail. Finally cation binding properties of DNA will be taken up with interest focussed mainly on the interaction of Mg^{2+} and DNA as manifested by ^{23}Na NMR.

3. THEORETICAL ASPECTS

For a nucleus with a spin quantum number greater than $\frac{1}{2}$ the NMR relaxation is usually dominated by the interaction between the nuclear electric quadrupole moment and the electric field gradient which are mathematically described by tensors. The nuclear quadrupole moment tensor can be expressed in terms of a single parameter (eQ) describing the deviation of the nuclear charge distribution from spherical symmetry. If the electric field gradient tensor is constructed in a principal coordinate system it reduces to two independent parameters:

$$eq \equiv V_{zz} \text{ and } \eta \equiv \frac{V_{xx} - V_{yy}}{V_{zz}} \quad (0 \leq \eta \leq 1)$$

where V_{ii} is a second derivative of the electrostatic potential and η is called the asymmetry parameter (η is zero in the case of axial symmetry). Here eq is the electric field gradient.

The strength of the interaction is usually described in terms of the quadrupole coupling constant $\chi \equiv \frac{e^2 q Q}{h}$. The nuclear

quadrupole (eQ) moment can be obtained from atomic beam experiments in combination with quantum mechanical calculations. However, a complication arises from the fact that the electrons around the nucleus are distorted from spherical symmetry as a result of charges outside the closed inner shells of the atom. This leads to a correction term in V_{zz} called the Sternheimer antishielding factor and thus we write $V_{zz} = V_{zz}^0 (1 - \gamma_\infty)$. Indeed this effect is found to be very important as for $^{35}\text{Cl}^-$ γ_∞ is -49 - -83 and for $^{23}\text{Na}^+$ γ_∞ is ca. -5 .¹ If the quadrupolar nucleus resides at a site with cubic symmetry, the electric field gradient cancels and the quadrupole coupling constant will be zero. Such is the case for ^{23}Na in crystalline NaCl or a tetrahedral molecule in the gaseous state.

In the liquid state, e.g. in aqueous solution, one has to take into account the time fluctuations of both the magnitude and direction of the field gradient. These fluctuations arise as other ions and dipoles in the solution move about changing the electronic distribution around the relaxing ion.

The general equations describing the quadrupolar relaxation of a nucleus with spin 3/2 have been derived by Hubbard.² Both the longitudinal and transverse relaxation are the sums of two exponential decays. Thus the longitudinal magnetisation is

$$\frac{M_L^O - M_L(t)}{M_L^O} = \frac{4}{5} \exp(-a_1 t) + \frac{1}{5} \exp(-a_2 t) \quad (1)$$

and the transverse magnetisation, in a coordinate system rotating at the Larmor frequency, is

$$\frac{M_T(t)}{M_T(0)} = \frac{3}{5} \exp(-b_1 t) + \frac{2}{5} \exp(-b_2 t) \quad (2)$$

a_1 , a_2 , b_1 and b_2 are given by

$$\begin{aligned} a_1 &= 8\pi^2 \left(\frac{eQ}{h}\right)^2 J_{-22}(2\omega) \\ a_2 &= -8\pi^2 \left(\frac{eQ}{h}\right)^2 J_{-11}(\omega) \\ b_1 &= 4\pi^2 \left(\frac{eQ}{h}\right)^2 (J_{00}(0) - J_{-11}(\omega)) \\ b_2 &= 4\pi^2 \left(\frac{eQ}{h}\right)^2 (J_{-22}(2\omega) - J_{-11}(\omega)) \end{aligned} \quad (3)$$

where ω is the Larmor frequency of the relaxing nucleus and J_{-kk} are the Fourier transforms of the time correlation function (C_{-kk}) of the electric field gradient. $C_{-kk}(t) = \overline{V_k(0) V_{-k}(t)}$, $J_{-kk}(\omega) = \frac{1}{2} \int_{-\infty}^{\infty} C_{-kk} \exp(i\omega t) dt$. Note that the field gradient is here defined in a non-principal coordinate system.

For a detailed understanding of the quadrupolar relaxation one has to set up a model for the electric field gradient taking into account ion-solvent and ion-ion interactions. A few such models are in use and for a discussion of these the reader is referred to the reviews by Lindman and Forsén.^{3,4} The electrostatic model of Hertz is discussed below in connection

with the relaxation of perchlorate.

If the field gradient tensor is described by the parameters eq and η and the correlation function is expressed as a single exponential then²

$$J_{-kk}(\omega) = (-1)^k \cdot \frac{1}{20} \cdot (1 + \frac{1}{3} \eta^2) (eq)^2 \tau_c [1 + \omega^2 \tau_c^2]^{-1} \quad (4)$$

where τ_c is the correlation time. In the case of extreme narrowing ($\omega^2 \tau_c^2 \ll 1$) the decay of the longitudinal and transverse magnetisations become equal and can be written as ($\eta \approx 0$)

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{2\pi^2}{5} \chi^2 \tau_c \quad (5)$$

This equation can be used directly without a quantitative understanding of either the quadrupole coupling constant or the correlation time, indeed a separation of these quantities is usually not possible from NMR measurements.

When the quadrupolar ion is bound to a macromolecule the extreme narrowing approximation is no longer valid. Bull⁵ has derived the equations for a nucleus with spin 3/2 nucleus exchanging between two sites. If one of these sites is assumed to be an ion binding site on a macromolecule (site B), and the other is the ion in bulk solution (site A), where the extreme narrowing condition applies, then a_1 , a_2 , b_1 and b_2 in eq. (3) are (under condition of fast exchange, see below)

$$a_1' = P_F a_{1A} + P_B a_{1B}, \quad a_2' = P_F a_{2A} + P_B a_{2B}, \quad (6)$$

$$b_1' = P_F b_{1A} + P_B b_{1B}, \quad b_2' = P_F b_{2A} + P_B b_{2B}$$

where P_F is the fraction of ions free in solution and P_B the fraction bound to the macromolecule. a and b for the A sites are given by equation (5) whereas those for the B site are given by equations (4) and (3). The decay of the magnetisation observed is thus an average of the effects of the free and bound ions. Generally, information on the bound site is carried over to the free site by exchange. If the

exchange time is very long on the NMR timescale (slow exchange), no information will be carried over from the bound ion to the bulk solution. In the case when the exchange time is very short (fast exchange) average effects as in equation (6) will be observed. Between these limiting cases more complex exchange conditions will apply. In most of the cases discussed in this thesis fast exchange conditions prevail and therefore the case of slow exchange will be omitted.

Usually a and b are, for bound ions, very much greater than for free ions, and therefore one can obtain large effects in the relaxation even when $P_B \ll 1$. a_1, a_2, b_1 and b_2 for the bound ions contain information on both the correlation time (τ_c) which can contain contributions from different types of motion, such as overall macromolecular rotation, chemical exchange and internal motion (the latter being discussed in [VII]), and quadrupole coupling constants (χ) for these bound ions. To obtain these is a matter of technique. Thus when $\omega\tau_c$ is not too large (in practice $\omega\tau_c < 1.5$) the relaxation equations (1) and (2) can be approximated as one exponential and the correlation time can be obtained from the ratio between the effective longitudinal and transverse relaxation times. The correlation time can also be obtained by fitting the transverse relaxation to two exponentials, or by fitting the spectrum (obtained by taking the Fourier transform of the free induction decay) to two Lorentzians. Finally, the frequency dependence of the longitudinal relaxation may be studied to yield τ_c . Once the correlation time is obtained, the product $P_B\chi^2$ is readily calculated and if P_B is known χ is obtained.

Much information can be obtained from the NMR relaxation of ions bound to macromolecules. For proteins the addition of a ligand may lead to a decrease in P_B and often the number of strong binding sites can be extracted. One can thus learn something both about specific and secondary binding sites. Upon varying the ion concentration information about the ion binding constant is obtained. The correlation time can be used to detect conformational changes in the macromolecule, and the quadrupole coupling constant may give information about the nature of the ion binding sites. Thus it has been observed that for Cl^- bound to metal ions embedded in a protein, χ is marked-

ly greater than for Cl^- bound to positively charged amino acid residues.⁶ Finally, it should be pointed out that although the equations above consider only one type of B site, the extension to incorporate several types of site is straightforward.

4. THE RELAXATION OF ClO_4^-

The most popular model for description of the relaxation of quadrupolar ions in solution is the electrostatic model of Hertz and coworkers described in a recent series of papers.⁷⁻¹¹ For additional reviews, see Refs. 3 and 4. In the electrostatic model, surrounding solvent dipoles and other ions, treated as point charges, set up time fluctuating field gradients causing the relaxation. The "fully random distribution" (FRD) model of Hertz, in which the solvent dipoles are assumed to have both random distribution and orientation relative to the relaxing ion, that is there is no distinct solvation sphere, appears to account quite satisfactorily for the relaxation of quadrupolar ions showing "structure-breaking" effects (e.g. Cl^- , Br^- , I^- , Rb^+ , K^+) in the limit of infinite dilution. Structure breaking ("negative hydration") means that the thermal motions of the water molecules are faster in the presence of such ions. As the perchlorate ion is strongly structure-breaking it would be expected that the FRD model is applicable to this ion. For a discussion of the structure-breaking phenomenon, see Ref. 12. In the FRD model the relaxation equations applying to nuclei with spin 3/2 are

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{32\pi^3}{5} \left(\frac{eQ\mu(1 - \gamma_\infty)P}{4\pi \epsilon_0 h} \right)^2 \frac{C_{\text{H}_2\text{O}} \tau_{\text{H}_2\text{O}}}{r_0^5} \quad (7)$$

when written in SI units. (Equation (1) in [1] lacks a factor 4 in the numerator, this does not, however, influence the calculations in the paper.) In equation (7) μ is the dipole moment of the solvent, C the solvent concentration (m^{-3}), τ the correlation time of pure solvent ($\tau_{\text{H}_2\text{O}} = 2.5 \cdot 10^{-12} \text{s}$) and r_0 is the distance between the relaxing ion and an adjacent solvent molecule, taken as the sum of the ionic radius and the radius of the solvent molecule ($r(\text{ClO}_4^- - \text{H}_2\text{O}) = (2.84 + 1.4) \text{\AA}$). P is a polarisation factor which describes the polarisation of water molecules due to ions present. Hertz assumes this factor to be 0.5 throughout.

One of the problems which arise when discussing the relaxa-

tion of $^{35}\text{ClO}_4^-$ concerns the value taken for the Sternheimer antishielding factor (γ_∞). As in [I] a value for γ_∞ can be obtained from the observed relaxation rate but this approach is not entirely satisfactory if it is desired to test the FRD model. Contreras and Hertz¹³ have compared the infinite dilution relaxation rate of $^{35}\text{ClO}_4^-$ with that of $^{81}\text{Br}^-$ and $^{127}\text{I}^-$, and after corrections for different values of r_0 and I , they ascribe the difference entirely to differences in the Sternheimer factor. Thus the value $\gamma_\infty = 40$ is obtained. This method also has its disadvantages, as γ_∞ for Br^- and I^- must be known; but these may in fact differ from the value used by a factor of two.¹ The same argument can also be raised against this approach as to that of [I]. Contreras and Hertz, when discussing their results on perchlorate, conclude that¹³ "... we have no evidence which would conflict with the FRD model of hydration". However, this is of course no proof of the validity of the model.

When discussing the quadrupolar relaxation of ions, as the salt concentration is increased the general approach of the electrostatic treatment is to take into account the concentration dependence of $C_{\text{H}_2\text{O}}$ and $\tau_{\text{H}_2\text{O}}$ in equation (7) and add a term corresponding to direct ion-ion interactions. If this approach is found to give satisfactory agreement with experimental results it is then concluded that ion-water and water-water correlations are not important, otherwise a qualitative discussion of these indirect effects are included. The equation put forward by Hertz then reads ($I = 3/2$)

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{32\pi^3}{5} \left(\frac{eQ(1 - \gamma_\infty)P}{4\pi\epsilon_0 h} \right)^2 \left(\frac{\mu^2 C_{\text{H}_2\text{O}} \tau_{\text{H}_2\text{O}}}{r_0^5} + \frac{2 C_{\text{ion}} e^2}{9 a \bar{D}} y\left(\frac{a}{\alpha}\right) \right) \quad (8)$$

$$y\left(\frac{a}{\alpha}\right) = \frac{1 - \frac{1}{2}\left(\frac{a}{\alpha}\right) + \frac{1}{2}\left(\frac{a}{\alpha}\right)^2 - \frac{1}{2}\left(\frac{a}{\alpha}\right)^3 \exp\left(\frac{a}{\alpha}\right) \cdot \int_{\frac{a}{\alpha}}^{\infty} \frac{e^{-s}}{s^3} ds}{1 + 4 \left(\frac{a}{\alpha}\right)^2}$$

New parameters are C_{ion} which is the ion concentration (m^{-3}), $\bar{D}$ is the mean self-diffusion coefficient of anion and cation, a is the distance of closest approach between the ions and α is the thickness of the ionic cloud around a given ion. If the

distance between a relaxing ion and another particular ion is greater than α , the given ion will not produce a field gradient at the relaxing ion. In the equation most of the parameters can be obtained from independent experiments and by making a reasonable guess of α (the ion-cloud parameter), α can be calculated from the observed relaxation rate. In their analysis of the perchlorate ion relaxation rates, Contreras and Hertz¹³ obtain $\alpha \approx 1.2$ Å for NaClO_4 and $\alpha \approx 1.0$ Å for LiClO_4 , approximately what might be expected, hence indirect effects seem to be of little importance.

It is thus found that the electrostatic approach gives satisfactory account of the relaxation of the perchlorate ion and that no need to introduce distortion of the ion from its tetrahedral symmetry arises in accordance with the conclusions in [I]. Finally, it should be noted that the results in [I and II] are in general agreement with those later reported,¹³⁻¹⁵ and that in the presence of paramagnetic ions such as Mn^{2+} the relaxation mechanism may no longer be the quadrupolar one [III]. As shown in [III] the relaxation in this case is mainly caused by the unpaired electron spin of Mn^{2+} and by studying the frequency and temperature dependences of the $^{35}\text{ClO}_4^-$ and $^{23}\text{Na}^+$ relaxation, information is obtained on the interionic distances as well as the rate of exchange between the Mn^{2+} -ion complex and the bulk solution.

5. THE ROLE OF IONS IN BIOLOGICAL SYSTEMS

The importance of ions and ionic interactions in biological systems is well documented. Most physiological processes take place in 0.1-0.15 M electrolyte solutions, in which the major simple ions are Na^+ , K^+ , Mg^{2+} , Ca^{2+} and Cl^- . Some roles for these and other simple ions are:

- a) maintaining the osmotic pressure on either side of cell membranes,
- b) activating and stabilizing a large number of enzymes,
- c) maintaining the sensitivity of nerves, and necessary for nerve impulse transmission, and
- d) control of muscle, and an important role in blood coagulation.

In addition to these specific roles, the relatively weak interactions of ions with charged groups or polar residues on the surface of a biological macromolecule are of importance in determining its conformation and biological activity. Also ionic interactions are important for the binding of coenzymes to enzymes, the specific interaction of subunits in a multisubunit protein, the interaction between histones and DNA in nucleosomes and so forth. Many highly charged biopolymers such as nucleic acids and mucopolysaccharides show a pronounced polyelectrolyte behaviour and as such their behaviour can often be understood on the basis of relatively simple theoretical considerations.

Figure 1 is a schematic diagram of a "generalised" cell illustrating some of the ionic interactions which take place.

It is of importance to study these ionic interactions on a molecular level and here NMR is a most useful method. There are several techniques in use.

One is to look directly at ions bound to macromolecules, here $^{113}\text{Cd}^{2+}$ ($I = \frac{1}{2}$) NMR is becoming increasingly popular to probe metal binding sites. Often different NMR signals are seen from $^{113}\text{Cd}^{2+}$ bound to different sites and valuable information concerning the binding, stoichiometry and the environment of the ion can be obtained.

Then there are indirect methods, such as to look at the ^1H , ^{13}C and ^{15}N NMR spectra of biological macromolecules and study

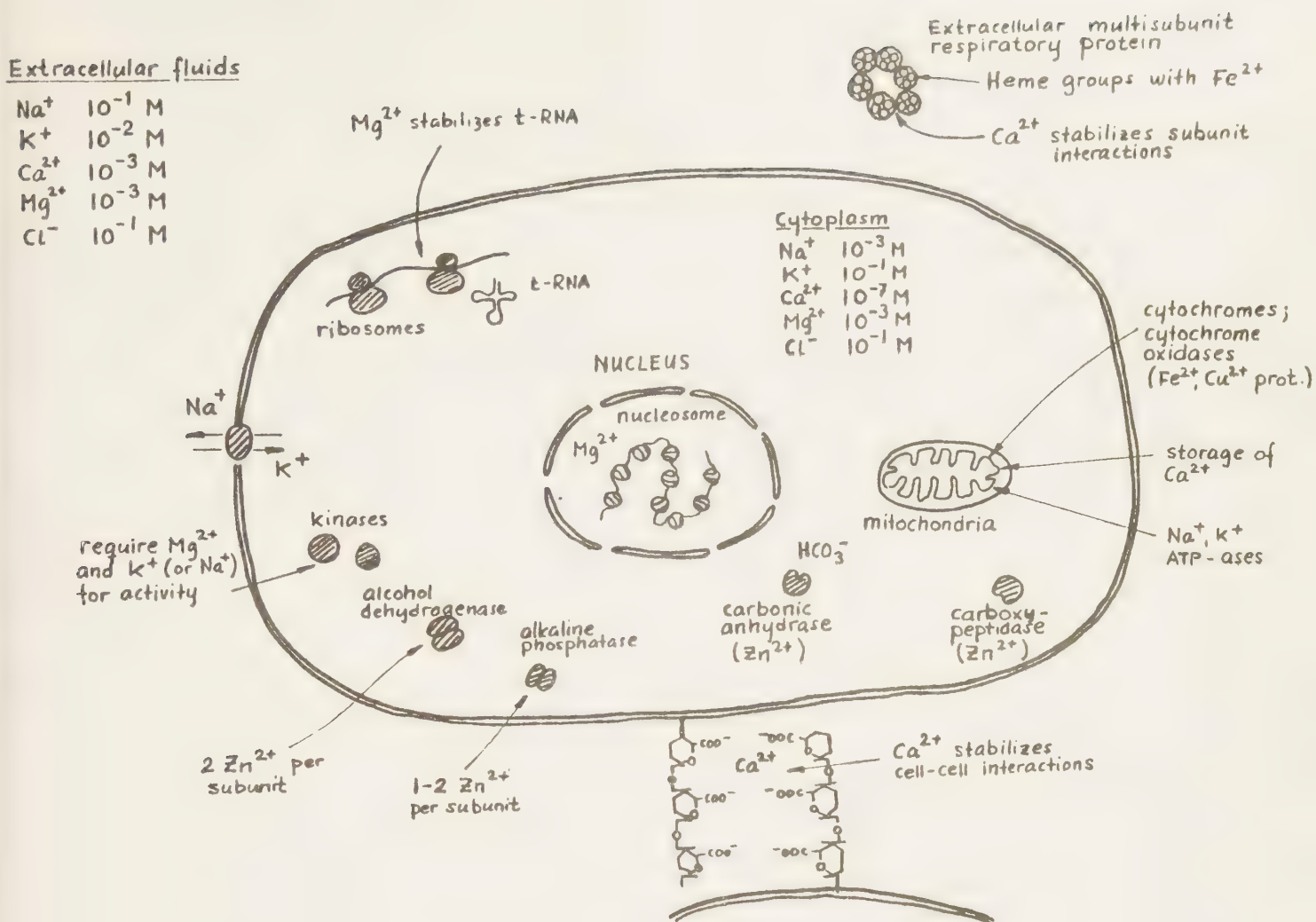


Figure 1. Schematic illustration of the role of ions and ionic interactions in a cell. (Courtesy of S. Forsén.)

spectral changes produced by the presence of different ions. The effect of bound paramagnetic ions on the water-proton relaxation rates has been successfully applied to the study of metal binding sites on biological macromolecules. Finally quadrupolar ions ($I > \frac{1}{2}$) can be used to probe ion binding in biological systems. It is usually not possible to look directly at the bound ions, the quadrupolar relaxation being so efficient that the NMR signal is broadened beyond detection. Instead, an excess of ions is used, the effect of the bound ions being mediated to the bulk ions by chemical exchange. This is the approach used in this thesis.

The following areas of application can be considered:

- a) The use of anions and cations as probes in the functional study of biological macromolecules,
- b) the identification and characterisation of ion binding sites,
- c) the relative binding strength of different ions and
- d) the testing of theories of ion binding to polyelectrolytes.

Various examples of these applications can be found in papers [IV-XIII], some of these are considered below.

6. THE BINDING OF MONOATOMIC IONS TO PROTEINS

6.1 Dehydrogenases

The structures of several nicotinamide-nucleotide-linked dehydrogenases have been determined to high resolution by X-ray techniques (for a review see Ref. 16). It has been found that there is a striking similarity in the structures of coenzyme binding domains of lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and liver alcohol dehydrogenase (LADH), whereas the catalytic domains have very different structures. The conformation of the bound coenzyme and its orientation and position on the proteins are all similar. Figure 2 is a schematic picture of the LDH:NAD⁺-pyruvate complex, and Figure 3 is a schematic drawing of the MDH:NAD⁺ complex.

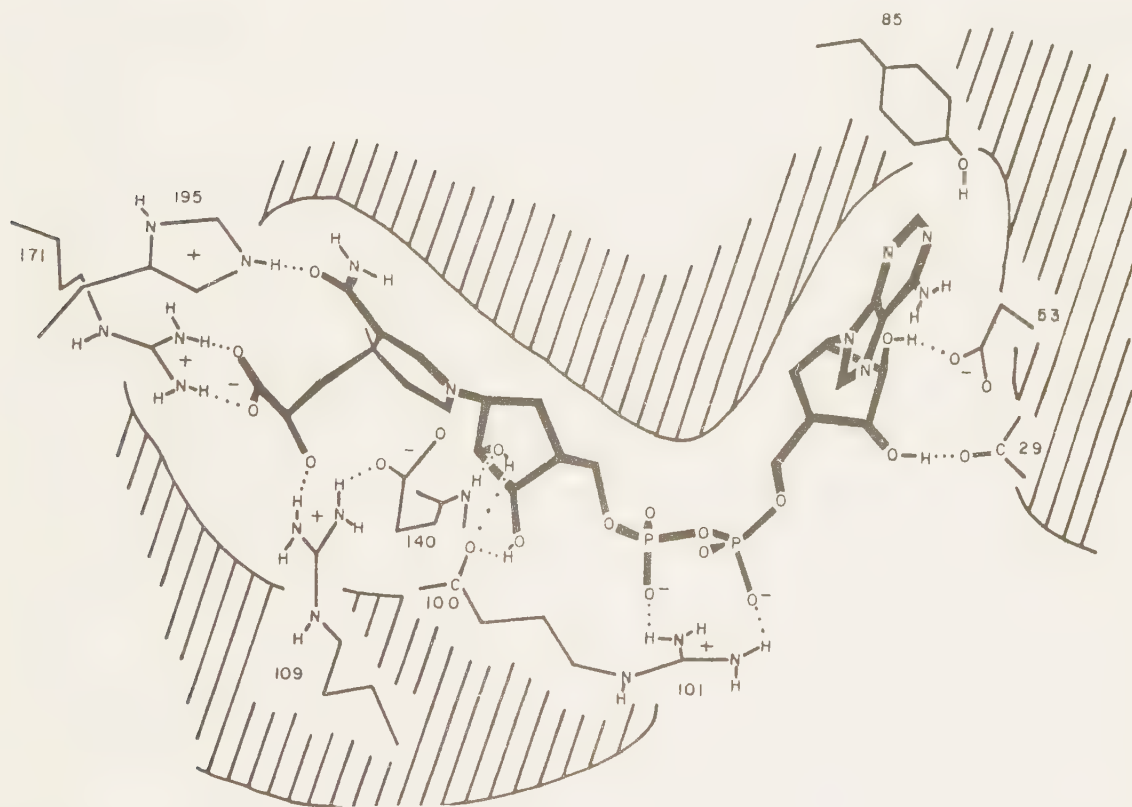


Figure 2. Schematic illustration of the LDH:NAD⁺-pyruvate complex. (From Adams, et al.¹⁷)

It is interesting to compare the effect caused by NADH on the ³⁵Cl⁻ relaxation for Cl⁻ bound to different dehydrogenases.

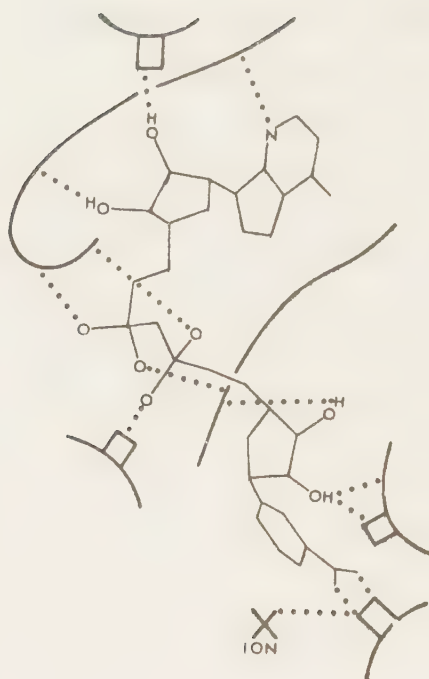


Figure 3. Schematic illustration of the MDH:NAD⁺ complex.
(From Webb et al.¹⁸)

Adding coenzyme to LDH produces an enhanced ³⁵Cl⁻ transverse relaxation, leaving the longitudinal relaxation unaltered, due to an increased correlation time τ_c [VI]. In the case of LADH, NADH binding leads to a stoichiometric release of Cl⁻ ions,¹⁹ as manifested by the decrease in the relaxation rates. For MDH the addition of NADH produces an enhancement in both the transverse and longitudinal relaxation [VII], implying either an increased binding or a larger quadrupole coupling constant. As the chloride ions bind at the active sites of the enzymes, these results clearly reflect the differences in the catalytic domains of the different dehydrogenases.

The increase in the correlation time of Cl⁻, bound at the substrate binding site of LDH, upon addition of NADH indicates an ability of bound coenzyme in restricting the motion at the substrate binding site.

These results, and results of ³⁵Cl⁻ NMR studies of ion binding to other proteins, have been analysed in terms of a simplified model for internal motion [VII]. In this model the chloride ion is bound to the protein by means of a rigid rod, and it is then allowed to diffuse back and forth in a conical

hole. The analysis shows that it is the size of this conical hole which decreases upon addition of coenzyme to LDH. This correlates well with the results of X-ray crystallography, where it has been shown that the guanidium group of Arg-109 moves into the active site anion binding region, upon addition of NADH.

6.2 Halophilic proteins

Halophilic bacteria are an example of life's adaptability to harsh environmental conditions, leading a normal and healthy life in more than 2 M sodium chloride solutions. The internal salt concentration of these bacteria often exceed their external concentrations, the total internal concentration of monovalent salt reaching up to about 5 M. This requires the biological processes to adapt to this saline environment, in particular all proteins must be able to function at conditions, which often would cause non-halophilic proteins to be salted-out, and cause multisubunit enzymes to dissociate rendering them inactive. Indeed, it is found that proteins isolated from halophilic bacteria require high concentration of salt for both optimum stability as well as activity.

An interesting feature of the halophilic proteins is that they carry a large excess of negative charges, for example ferredoxin (FD) from halophilic bacteria has about 40 negative charges and only 6-7 positive ones.

The ion binding properties of the halophilic proteins are therefore of considerable interest and here NMR studies of ion binding to the proteins might assist in deducing a molecular basis for halophilism. In [X] it is shown that the anion binding of halophilic ferredoxin may be profitably explored, and that in spite of the large excess of negatively charged groups, several anion binding sites are retained.

6.3 Parvalbumin

Parvalbumins are a class of small, water soluble proteins which are ubiquitous in muscle. They contain two tightly bound calcium ions per protein molecule and have an high content of phenylalanine and alanine residues. The two Ca^{2+} ions occupy specific sites, and are coordinated by six oxygen atoms with an octahedral arrangement around the central cation.

It is shown in [X] that sodium binds only weakly to parvalbumin isolated from the white muscle of hake, and that there is no competition between Ca^{2+} and Na^+ . However, the presence of EGTA causes a marked enhancement of the $^{23}\text{Na}^+$ linewidth probably due to a tertiary complex between parvalbumin-EGTA and Na^+ . It is also shown that Ca^{2+} competes effectively with Na^+ for these additional binding sites.

It is thus clear that, when metal complexing agents such as EGTA or EDTA are used in preparation procedures, care must be exercised so that these do not affect the cation binding properties of proteins.

7. THE BINDING OF SYMMETRICAL ANIONS TO PROTEINS

Discussions on the general methodology of NMR studies of ion binding to macromolecules can be found in chapters 8 and 9 of Ref. 3, as well as in other reviews by Forsén and Lindman.^{20,21} In [IV and V] the use of symmetrical anions to probe anion binding to proteins is discussed. Therefore the discussion here will be limited mainly to comparison of chloride and perchlorate binding to some proteins; some theoretical ^{19}F spectra of AsF_6^- will also be presented.

It is expected that the use of (rather large) symmetrical anions (e.g. ClO_4^- , AsF_6^-) can give complementary information on protein anion binding sites, because of their low tendency to coordinate to metal ions. One point to emerge, however, is that at least for ClO_4^- the relaxation effects observed are usually much smaller than those for Cl^- , even though ClO_4^- , according to the lyotropic series, binds more strongly to proteins than Cl^- . Thus when comparing results on human serum albumin (HSA) from [IV] and Ref. 22 it is found that the ratio

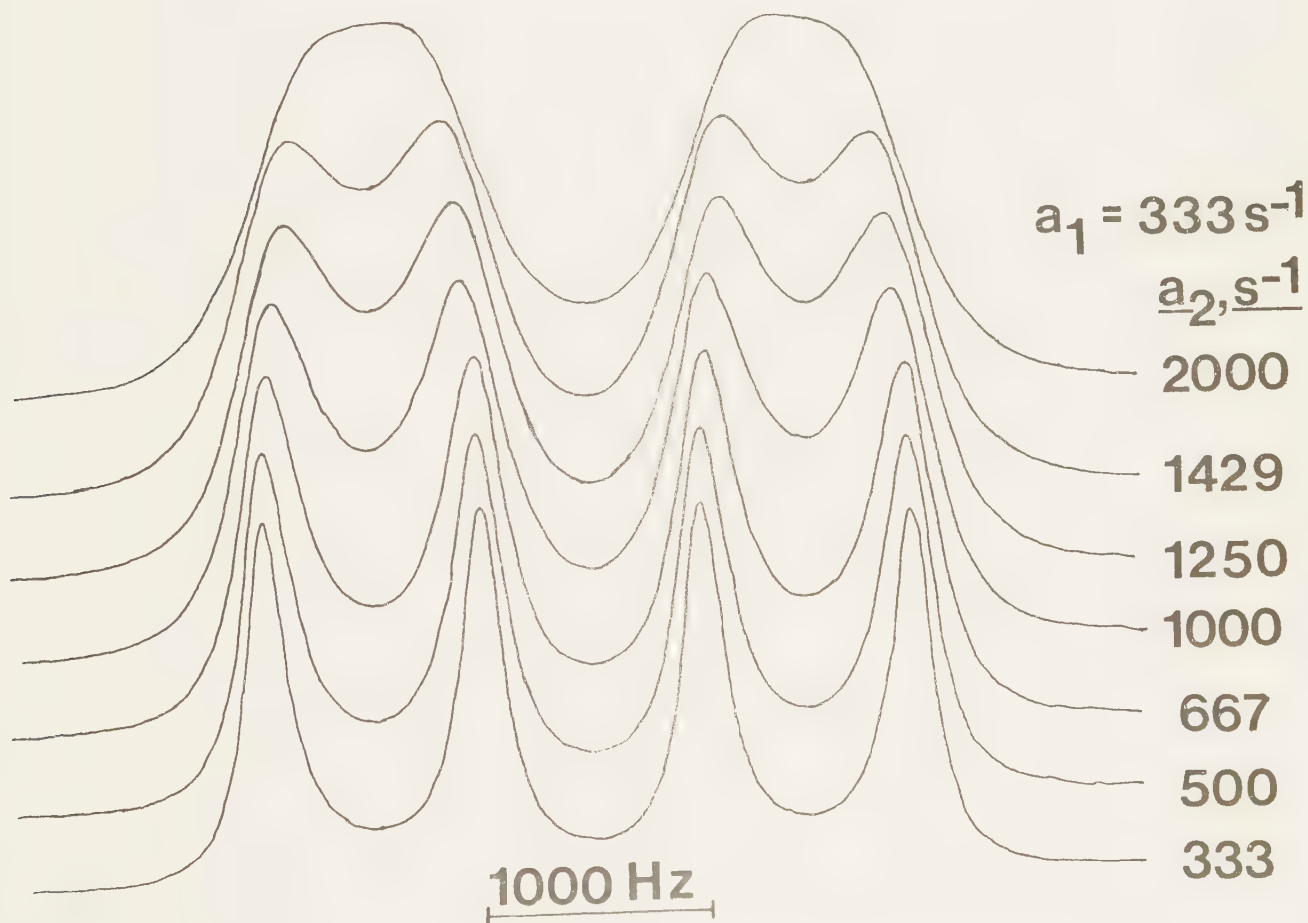
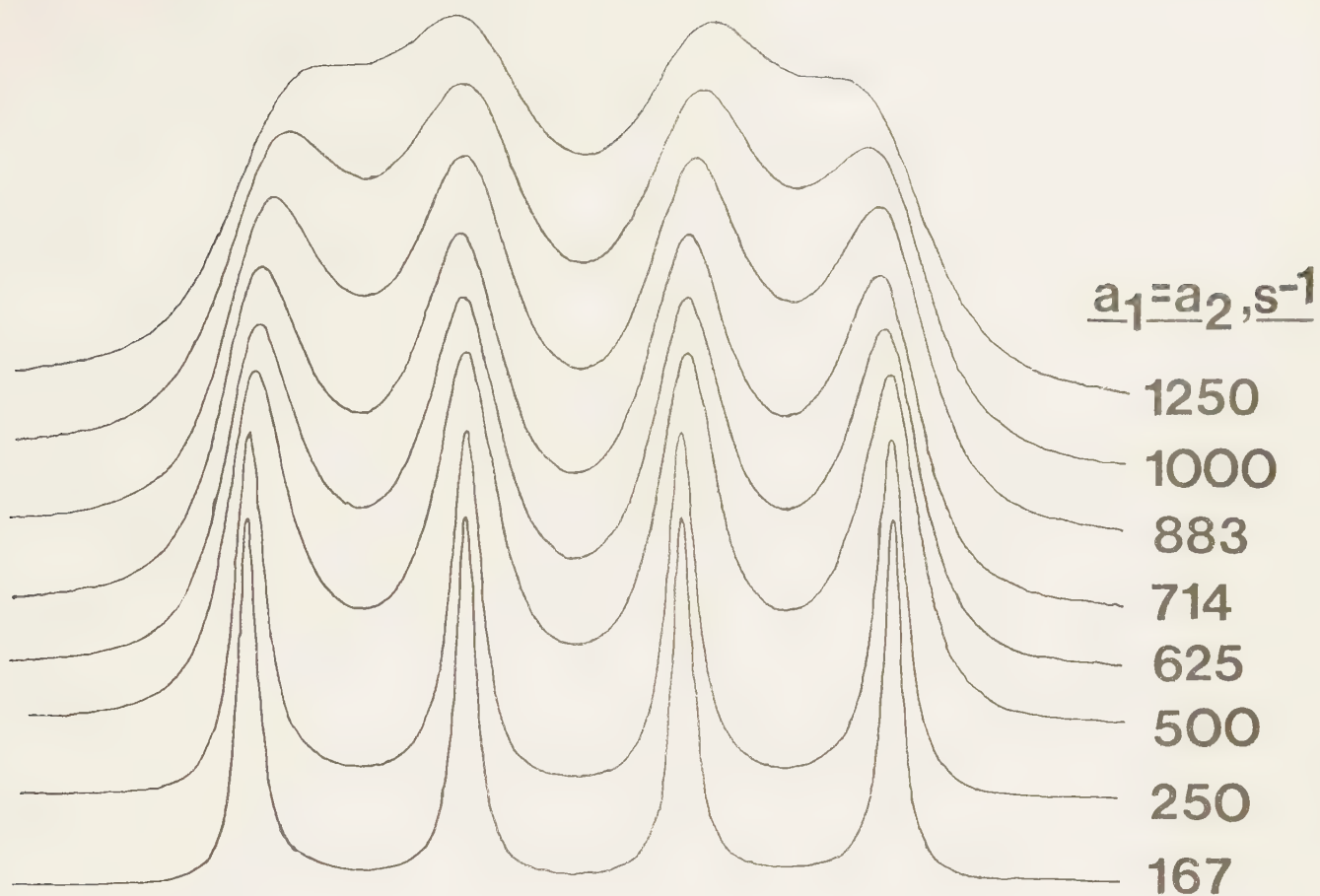
$$\left(\frac{1}{T_2} - \frac{1}{T_2^0}\right)_{\text{ClO}_4^-} / \left(\frac{1}{T_2} - \frac{1}{T_2^0}\right)_{\text{Cl}^-} \approx 0.2$$

(solution composition etc. being similar); this difference must therefore arise from differences in the quadrupole coupling constant (χ) (the correlation times do not differ by very much [IV], Ref. 20). To obtain the ratio between the quadrupole coupling constants, the ratio between the relaxation rates can be obtained for only the strong anion binding sites on HSA, and the value obtained is 0.1.^{22,23} This difference can be caused by different Sternheimer factors ($\gamma_{\text{Cl}}/\gamma_{\text{ClO}_4} \approx 1-3$) or as discussed in [I] the field gradient sensed by the chlorine atom in ClO_4^- is smaller than in Cl^- . Similar results were obtained by Rose and Bryant on HSA²⁴ and studies on other proteins (e.g. alkaline phosphatase and lactate dehydrogenase)²² indicate that the ratio between the relaxation of ClO_4^- and Cl^- is 0.1-0.3.

However, there is no rule without some exceptions. Andersson et al. have studied the binding of both Cl^- and ClO_4^- to both

the reduced and oxidised form of cytochrome C.^{25,26} In general they found that the linebroadening observed with Cl^- is much larger than that for ClO_4^- , however, at $\text{pH} \approx 9$ the linewidth of the perchlorate anion is markedly increased in the presence of the oxidised form of the protein and becomes comparable to that of Cl^- . These results are interpreted in terms of an increased binding of ClO_4^- and also paramagnetic effects may be present.²⁶ AsF_6^- is expected to bind in a similar fashion as ClO_4^- , however, it has an advantage that ^{19}F spectra can be recorded and as the ^{19}F nucleus has a higher sensitivity and is more abundant than ^{35}Cl the ^{19}F spectra can be obtained at lower ionic concentrations. The ^{19}F spectrum of AsF_6^- is a quartet, due to the spin coupling to ^{75}As ($I = 3/2$) and the relaxation mechanism is the scalar relaxation of the second kind. Information is thus obtained about the relaxation of the central nucleus, and as measurements of the $^{35}\text{ClO}_4^-$ relaxation times are often subject to experimental difficulties, AsF_6^- may be profitably used to explore protein anion binding sites.

The spectrum is characterised by three parameters; the spin coupling constant ($J_{^{75}\text{As}-^{19}\text{F}} = 940 \text{ Hz [V]}$), and the two rate constants a_1 and a_2 in equation (6). These constants are called $P_{m,m+2}$ and $P_{m,m+1}$ in [V] where it is also shown that they are very sensitive to the HSA concentration. On the following page, a few calculated spectra are presented, in the upper part $a_1 = a_2$, i.e. the extreme narrowing condition applies; in the lower part a_1 and a_2 are different and an increase in a_2 corresponds to a larger correlation time. The upper half of the figure is in essence the same as that published in Refs. 27 and 28.



8. CATION BINDING TO DNA

A fundamental property of nucleic acids is their high electric charge, due to ionised phosphate groups. This property makes the interaction of nucleic acids and cations in general to be of primary importance in maintaining the structural stability, and in controlling the biological function of nucleic acids. Metal ions are found to be associated with nucleic acids in the natural environment (for a review see Ref. 29). Polyamines are generally found in biological materials and have a variety of effects on nucleic acid biosynthesis and metabolism in vitro (for a review see Ref. 30). Finally proteins with a large excess of positive charges (histones) are found to be condensed with DNA in chromatin (for a review see Ref. 31), where DNA is periodically folded by a complex of histones, creating a string of specific DNA-protein complexes called nucleosomes.

Manning³² has reviewed the ion binding properties of polynucleotides in terms of polyelectrolyte behaviour and has been able to rationalise a large body of data in terms of simplified electrostatic theory. Central to Manning's model is the concept of "counterion condensation", which occurs above a critical linear charge density, and which states that the "charge fraction" of the polyelectrolyte is constant over a broad concentration range. Thus the charge fraction of double stranded DNA is 0.24 and 0.12 in the presence of Na^+ and Mg^{2+} , respectively.

In [XI] the interaction of DNA and Na^+ is studied and the dynamic behaviour of the sodium ions in the presence of both single- and double stranded DNA as well as the quadrupolar coupling constants are discussed. In [XII] it is shown that both $^{25}\text{Mg}^{2+}$ and $^{43}\text{Ca}^{2+}$ can be useful for the study of DNA-cation interaction and in [XIII] ^{23}Na NMR has been used to study the dissociation of histones from the chromatin complex.

The discussion is now focussed on the $^{23}\text{Na}^+$ NMR signal, in the presence of DNA, and on what happens when competing cations (Mg^{2+} and different polyamines) are added to the system.

Figure 4 shows the excess $^{23}\text{Na}^+$ linewidth as a function of

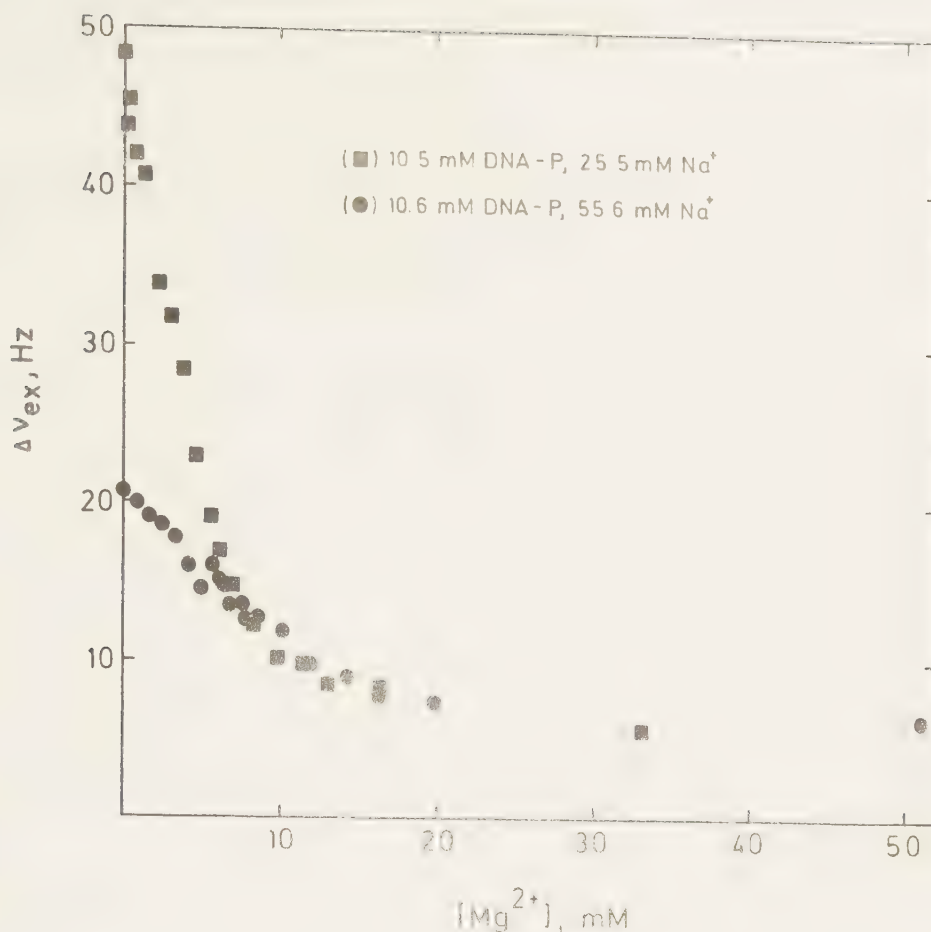


Figure 4. The excess ^{23}Na linewidth as a function of added Mg^{2+} for aqueous DNA solutions. (■) 10.5 mM DNA-P and 22.5 mM Na^+ , (●) 10.6 mM DNA-P and 55.6 mM Na^+ . The solutions were buffered with phosphate at pH 6.8, temperature $4 \pm 1^\circ\text{C}$. The spectra were obtained at 2.3 T (XL-100 NMR spectrometer).

Mg^{2+} concentration at two different sodium concentrations. The first point to be noted is that according to the ion condensation model, the fraction of ions condensed is inversely proportional to the concentration of salt, and the same should therefore be expected for the linewidth. With only two points this seems approximately true. In Ref. 33 an expression is given for the binding of Mg^{2+} to DNA in the presence of excess 1:1 salt. Application of this expression to the results in Figure 1 gives a titration curve which is too steep at low Mg^{2+} concentrations, but which is in qualitative agreement as it predicts Mg^{2+} to be preferably associated to DNA at low Na^+

concentrations as seen in Figure 4. In order to rationalise these data, efforts are currently being made to obtain theoretical curves for the number of monovalent cations within a given distance from the DNA cylinder, by solving the Poisson-Boltzmann equation.³⁴ Further discussions will therefore not be presented here.

The titration of aqueous solutions of DNA with various polyamines (di-, tri- and tetraamines) has a profound effect on the excess $^{23}\text{Na}^+$ linewidth,³⁵ the most important feature of the titration curves is a sharp kink when the number of added positive amino groups equals that of DNA-phosphates, indicating that one amino group binds stoichiometrically to one phosphate group. However, drastic changes are produced in the binding properties of the amines upon varying the chain length between the amino groups $(\text{NH}_3^+-(\text{CH}_2)_4-\text{NH}_2^+(\text{CH}_2)_3-\text{NH}_3^+)$ being much more effective in expelling Na^+ than $\text{NH}_3^+-(\text{CH}_2)_3-\text{NH}_2^+(\text{CH}_2)_3-\text{NH}_3^+)$.

These examples are included to show the potential use of $^{23}\text{Na}^+$ NMR when studying cation binding to nucleic acids.

Finally, some examples where it is expected that the use of quadrupolar ions will prove valuable will be mentioned.

More detailed studies of the interaction between polyamines and DNA might give information on the mode of binding, e.g. do the polyamines bind to the base pairs of DNA as suggested in Ref. 36, and is the model of Liquori et al. for the binding of spermine and spermidine to DNA adequate?^{37,38}

Cation binding to transfer RNA can certainly be explored in a similar way as to DNA. An interesting aspect is whether tRNA behaves similarly to DNA and what differences are noticed. More detailed studies of chromatin and nucleosomes are certainly warranted and specifically, the question should be asked, whether the sodium binding properties of chromatin are affected by the EDTA used in the preparation procedure of [XIII]. On the basis of the results in [X] a rather important effect can be envisaged.

Histones, carrying a large excess of positive charges, show an increase in intermolecular association when salt concentration is increased, as well as changes in intramolecular structure. Here $^{35}\text{Cl}^-$ could answer questions on processes about which little is known at present.

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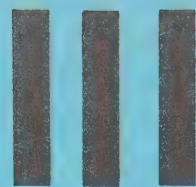
To you all I should like to recite the following lines from a poem by Bob Dylan:

May you grow up to be righteous
May you grow up to be true
May you have a strong foundation
When the winds of changes shift
May you always do for others
And let others do for you
May you stay forever young

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NMR STUDIES OF THE INTERACTION BETWEEN Mn^{2+} AND ClO_4^- IN
AQUEOUS SOLUTION

Pétur Reimarsson

Physical Chemistry 2, Chemical Center, P.O.B. 740,
S-220 07 Lund, Sweden

18 pages, 1 table, 4 figures.

Abstract

Longitudinal and transverse relaxation rates of ^1H , ^{35}Cl , and ^{23}Na in aqueous solution containing $\text{Mn}(\text{ClO}_4)_2$ and NaClO_4 have been studied as a function of temperature. The water proton relaxation rates are shown not to be affected by high concentrations of NaClO_4 . It is shown that the chlorine relaxation of ClO_4^- is dominated by the interaction with the unpaired electron spin of Mn^{2+} , and that ClO_4^- forms an inner sphere complex with Mn^{2+} . The rotational correlation time (τ_r) of ClO_4^- in this complex is $4.6 \cdot 10^{-11}$ s and the lifetime τ_M is less than $7 \cdot 10^{-9}$ s (300 K). The $^{23}\text{Na}^+$ relaxation is dominated by the Mn^{2+} - ^{23}Na electron-nucleus dipolar interaction and is not dependent on magnetic field. It is shown that Na^+ is probably separated from Mn^{2+} by one water molecule and a rotational correlation time $\tau_r > 7 \cdot 10^{-11}$ s (300 K) is obtained.

Introduction

Direct studies of ions in solution by nmr have been numerous in the past decades as reflected in a recent review [1]. Most of the ions have a spin quantum number greater than $1/2$ and thus the dominating relaxation mechanism is the quadrupolar one. The chlorine relaxation of the perchlorate anion has been subject to a number of studies both in aqueous solution and other solvents (for a review see [1,2]). In particular the presence of paramagnetic ions such as Mn^{2+} and Fe^{3+} has been found to have a great effect on the relaxation of $^{35}\text{Cl}^-$ and $^{35}\text{ClO}_4^-$ [3-7].

The effect on the ^{35}Cl linewidth of perchlorate has been attributed to an isotropic hyperfine interaction [4] and a weak inner sphere complex between the manganous ion and perchlorate was proposed. Another study suggested that the ^{35}Cl relaxation of ClO_4^- arises from electron-nucleus dipole-dipole relaxation but that the transverse relaxation was dominated by the slow exchange of ClO_4^- between the weak inner sphere complex with Mn^{2+} and the bulk solution [6]. From measurements of the water proton longitudinal relaxation rate the stability constant of MnClO_4^+ has been determined [8] as 2 M^{-1} . Paramagnetic ions can also affect the relaxation of other cations in the solution; thus Cs^+ has been found to be relaxed by Fe^{3+} [9] and Mn^{2+} [10,11]. The formation of a complex such as MnCl_2Cs^+ was found to explain the relaxation in [10]. The $^{23}\text{Na}^+$ relaxation rates in a $\text{MnCl}_2/\text{NaCl}$ solution have also been studied [12] where the relaxation mechanism of ^{23}Na was proposed to be dipolar and it was found that the distance of closest approach between Mn^{2+} and Na^+ is 4.5 Å.

Here we report the ^1H relaxation rates of a solution containing high concentration of NaClO_4 in presence of Mn^{2+} . It will be shown that the proton relaxation rates are not affected

by the high salt concentration to any great extent. We further report the ^{35}Cl (and ^{37}Cl) relaxation rates of ClO_4^- as function of temperature and frequency. The longitudinal relaxation rate is shown to be dominated a dipole-dipole interaction between the electron and nuclear spins whereas the scalar interaction contributes the greater part of the transverse relaxation. Finally the $^{23}\text{Na}^+$ relaxation rates as function of both frequency and temperature are presented; here both T_1 and T_2 are dominated by the dipolar interaction.

Theory

When a nucleus with magnetogyric ratio γ_I is relaxed by a paramagnetic center with total electron spin S the relaxation rates can be written as [13,14]

$$\frac{1}{T_{1M}} = \frac{2}{15} \left(\frac{\mu_0}{4\pi} \right)^2 g^2 \beta^2 S(S+1) \gamma_I^2 \frac{1}{r^6} \left[\frac{3\tau_c}{1+\omega_I^2 \tau_c^2} + \frac{7\tau_c}{1+\omega_S^2 \tau_c^2} \right] + \frac{2}{3} S(S+1) \left(\frac{A}{\hbar} \right)^2 \left(\frac{\tau_e}{1+\omega_I^2 \tau_e^2} \right)^2 \quad (1)$$

$$\frac{1}{T_{2M}} = \frac{1}{15} \left(\frac{\mu_0}{4\pi} \right)^2 g^2 \beta^2 S(S+1) \gamma_I^2 \cdot \frac{1}{r^6} \left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2 \tau_c^2} + \frac{13\tau_c}{1+\omega_S^2 \tau_c^2} \right] + \frac{1}{3} S(S+1) \left(\frac{A}{\hbar} \right)^2 \left(\tau_e + \frac{\tau_e}{1+\omega_S^2 \tau_e^2} \right) \quad (2)$$

where μ_0 is the vacuum permeability, β the Bohr magneton and r is the distance between the paramagnetic center and the relaxing nucleus. Here it is assumed that the g value is isotropic which seems to be applicable in most cases for Mn^{2+} [15] and that the electronic Larmor frequency (ω_S) is much larger than the corresponding nuclear frequency (ω_I). The first term in the equations arises from the modulation of the dipole-dipole interaction between the electronic and nuclear spins which is characterised

by a correlation time τ_c . The second term arises from the modulation of the scalar coupling between the spins which is characterised by the hyperfine coupling constant A/h and a correlation time τ_e . The correlation times can be written as $\tau_c^{-1} = \tau_r^{-1} + \tau_M^{-1} + \tau_S^{-1}$ and $\tau_e^{-1} = \tau_M^{-1} + \tau_S^{-1}$ where τ_r is the rotational correlation time of the complex, τ_M is the lifetime of the nucleus in the complex and τ_S is the electron spin relaxation time which will in general be frequency dependent [16]. It is assumed that there is only one electron spin relaxation time to consider. Furthermore, in the analysis below we shall assume that $\omega_I^2 \tau_c^2 \ll 1$ as τ_c will be dominated by τ_R which is of the order of 10^{-11} s. Also we shall assume that the second term in $1/T_{1M}$ is negligible and therefore also the term $\tau_e/(1+\omega_S^2 \tau_e^2)$ in the expression for $1/T_{2M}$. The relaxation rates measured are now given by the expression

$$\frac{1}{T_{iP}} = \frac{1-P_B}{T_{iO}} + \frac{P_B}{T_{iM} + \tau_M} \quad i=1,2$$

where P_B is the fraction of nuclei "bound to" the paramagnetic center and T_{iO} are the relaxation rates in the absence of paramagnetic ions. Below we report the excess relaxation rates

$$\Delta\left(\frac{1}{T_i}\right) = \frac{1}{T_{iP}} - \frac{1}{T_{iO}} \approx \frac{P_B}{T_{iM} + \tau_M}$$

as T_{iO}^{-1} are much smaller than T_{iP}^{-1} .

Experimental

All relaxation times were measured on a Bruker Bkr 322s pulsed spectrometer interfaced with a Varian V-71 computer using a homemade program. The longitudinal (T_1) relaxation times were measured using a $180^\circ - \tau - 90^\circ$ pulse sequence and the transverse

(T_2) relaxation times by means of the Meiboom Gill modification of the Carr-Purcell sequence. Each reported T_1 is the average of at least three and each T_2 the average of at least five independent measurements. The resulting errors are expected to be less than 5%. Homemade probes were used and the temperature was maintained by means of dry thermostated gas and is accurate to ± 0.5 K. Salts of analytical grade were used without further purification.

Results and Discussion

Water proton relaxation rates

Figure 1 shows the solvent water proton relaxation rates as a function of the inverse temperature at 61 MHz (1.403T) in a solution containing 6.28 M NaClO_4 and 6.54 mM Mn^{2+} . The various parameters in the equations describing the relaxation have been obtained for Mn^{2+} solutions previously [16-18]. It has also been shown that the presence of high concentration of NaClO_4 has very little effect on the water proton longitudinal relaxation rates [8]. We have applied the computer program described in [19] to obtain a best fit to our data at this one frequency. The results together with some literature values are shown in table 1. To obtain the best fit we chose to fix r and the coordination number in order to reduce the number of parameters. Another best fit could easily be obtained with a different value for q and slightly different r . The important point is however the similarity between our best fit parameters and those obtained by Bloembergen and Morgan [16]. In particular the high salt concentration does not seem to distort the hydration sphere of Mn^{2+} as this would probably be reflected in more efficient

electronic relaxation (τ_S would become shorter). Our best fit parameters have been used to draw the continuous curve in figure 1.

The chlorine relaxation rates of ClO_4^-

Figure 2 shows the excess ^{35}Cl and ^{37}Cl relaxation rates of ClO_4^- at the same magnetic field (2.07 T) as a function of the concentration of Mn^{2+} . The excess relaxation rates are the difference between the measured relaxation rates and those of a solution containing only NaClO_4 . It is seen that the relaxation rates are linearly dependent on the Mn^{2+} concentration and that the ratio of the relaxation rates $\Delta(\frac{1}{T_1})[^{35}\text{Cl}]/\Delta(\frac{1}{T_1})[^{37}\text{Cl}]$ is 1.4 thus demonstrating that the relaxation is caused by the paramagnetic Mn^{2+} ion. Both the dipolar and scalar terms of the paramagnetic relaxation equations are proportional to the square of the magnetogyric ratio γ_I whereas if the relaxation is quadrupolar then the rates are proportional to the square of the quadrupole moment, Q . For the chlorine isotopes we have $\gamma_I^2(^{35}\text{Cl})/\gamma_I^2(^{37}\text{Cl}) = 1.44$ and $Q^2(^{35}\text{Cl})/Q^2(^{37}\text{Cl}) = 1.61$ respectively. Furthermore figure 1 shows that there is a large difference between the longitudinal and transverse relaxation rates which displays the importance of the hyperfine interaction to the latter. Figure 3 shows the temperature dependence of the ^{35}Cl relaxation rates of ClO_4^- at the frequency 8.82 MHz (2.07 T) and also included are three points run at 5.98 MHz (1.403 T). It is noted that the relaxation seems to be independent of frequency thus indicating that $\omega_S^2 \tau_C^2 \gg 1$ (the other limit would give an unreasonable value for the correlation time τ_C). Now we have to make some assumption about P_B , the fraction of perchlorate "bound to" Mn^{2+} . Hertz and Keller [8] have obtained a value of 2 M^{-1} for the binding constant at 298 K which gives $P_B \approx [\text{Mn}^{2+}]/[\text{ClO}_4^-] = 0.25$. After insertion of numerical constants we can therefore write $\Delta(\frac{1}{T_1}) =$

$2.41 \cdot 10^{15} \cdot \frac{\tau_c}{r^6}$ where r is expressed in Ångström. If we take r as the sum of the ionic radii of ClO_4^- (2.84 Å) and Mn^{2+} (0.80 Å), i.e. we assume the formation of an inner sphere complex, one obtains the correlation time $\tau_c = 4.6 \cdot 10^{-11}$ s at 300 K which is equal to the rotational correlation time as both τ_M and τ_S are orders of magnitude larger.

The difference between the transverse and longitudinal relaxation rates can be written as

$$\Delta\left(\frac{1}{T_2}\right) - \Delta\left(\frac{1}{T_1}\right) \approx \Delta\left(\frac{1}{T_2}\right) - \frac{7}{6} \Delta\left(\frac{1}{T_1}\right) = 0.73 \left(\frac{A}{\hbar}\right)^2 \tau_e$$

and from figure 3 it is inferred that this difference increases as the temperature decreases. If τ_e were dominated by τ_S then the opposite behaviour would be expected as in our case τ_S decreases as the temperature is lowered as shown from the proton relaxation data [16]. Also as τ_S in general is frequency dependent, $\Delta(1/T_2)$ is expected to change with frequency if τ_S contributes markedly to τ_e . We therefore conclude that τ_e is dominated by the lifetime (τ_M) of ClO_4^- in the inner sphere complex. If we assume the same value for τ_S as obtained from the proton relaxation at this magnetic field (2.07 T) [16] then τ_M can be obtained as $\tau_M < 7 \cdot 10^{-9}$ s (300 K). This value is similar to that obtained for chlorine in FeCl^{2+} [9] and it also is clear that the case of slow exchange as applied in [6] is not valid in this case. The hyperfine coupling constant can now be obtained as $A/\hbar > 3.5 \cdot 10^5 \text{ s}^{-1}$. Further analysis of the curves is not feasible as τ_M , τ_r as well as P_B are temperature dependent and τ_S is expected to play a greater role as the temperature is lowered.

^{23}Na relaxation rates

Figure 4 shows the excess relaxation rates of $^{23}\text{Na}^+$ as a function of inverse temperature at three different magnetic fields (2.07, 1.75, 1.403 T). Here the excess relaxation rates are differences between the measured relaxation rates with manganese and those of a similar solution with magnesium where the quadrupolar relaxation dominates. It is seen that the relaxation rates are independent of frequency and that the ratio $\Delta(\frac{1}{T_2})/\Delta(\frac{1}{T_1}) = 1.18$ which indicates that the relaxation is dominated by dipolar mechanism with $\omega_S^2 \tau_C^2 \gg 1$ as then this ratio is expected to equal $7/6 \approx 1.17$. After insertion of numerical constants in the relaxation equations we get $\Delta(\frac{1}{T_1}) = \frac{6}{7} \Delta(\frac{1}{T_2}) = 7.02 \cdot 10^{16} \cdot \frac{\tau_C}{r^6} \cdot P_B$ where r is expressed in Ångström. The next question to ask is how the dipolar interaction is modulated from Mn^{2+} to Na^+ . If we assume a linear structure with the perchlorate situated between the cations ($r \approx 7.5$ Å) we obtain a correlation time $\tau_C > 1 \cdot 10^{-9}$ s (300 K) regardless of P_B . This value seems far too high as then the factor $\omega_I \tau_C$ is expected to come into play. If we instead assume that there is a water molecule between the cations then with $r \approx 4.7$ Å we obtain $\tau_C > 7 \cdot 10^{-11}$ s (300 K). These results are similar to those obtained from the $^{23}\text{Na}^+$ relaxation in $\text{NaCl}/\text{MnCl}_2$ solutions [12].

Conclusions

We have demonstrated the feasibility of a multinuclear nmr approach to study the paramagnetic relaxation of nuclei such as ^{35}Cl and ^{23}Na which are usually relaxed by quadrupolar relaxation. We have demonstrated that ClO_4^- forms an inner sphere complex with Mn^{2+} whereas Na^+ is probably separated from Mn^{2+} by one water

molecule. However, same caution should be exercised, as the high concentration of Mn^{2+} used may possibly require that the relaxation equations be modified.

Acknowledgements

I should like to express my sincere thanks to Dennis R. Burton, Björn Lindman and Gunnar Karlström for their advice. Mats Lundell kindly draw the figures.

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Table 1. Parameters for water protons in the hydration sphere of Mn^{2+} as obtained from proton relaxation rates at 61 MHz in a solution containing 6.28 M NaCl. Also shown are some results from the literature.

<u>Parameter</u>		<u>Best-fit value</u>	<u>ref. [16]</u>	<u>ref. [17]</u>
r	Å	2.77	2.77	-
Coordination number		6	6	-
A/ $\hbar$	s^{-1}	$5.4 \cdot 10^6$	$6.2 \cdot 10^6$	-
τ_r (300 K)	s	$4.3 \cdot 10^{-11}$	$3 \cdot 10^{-11}$	-
τ_M (300 K)	s	$1.5 \cdot 10^{-8}$	$2.3 \cdot 10^{-8}$	-
τ_S (300 K)	s	$7.8 \cdot 10^{-8}$	$1.8 \cdot 10^{-8}$	$2.4 \cdot 10^{-8}$
τ_e (300 K)	s	$1.3 \cdot 10^{-8}$	$1.0 \cdot 10^{-8}$	$1.4 \cdot 10^{-8}$

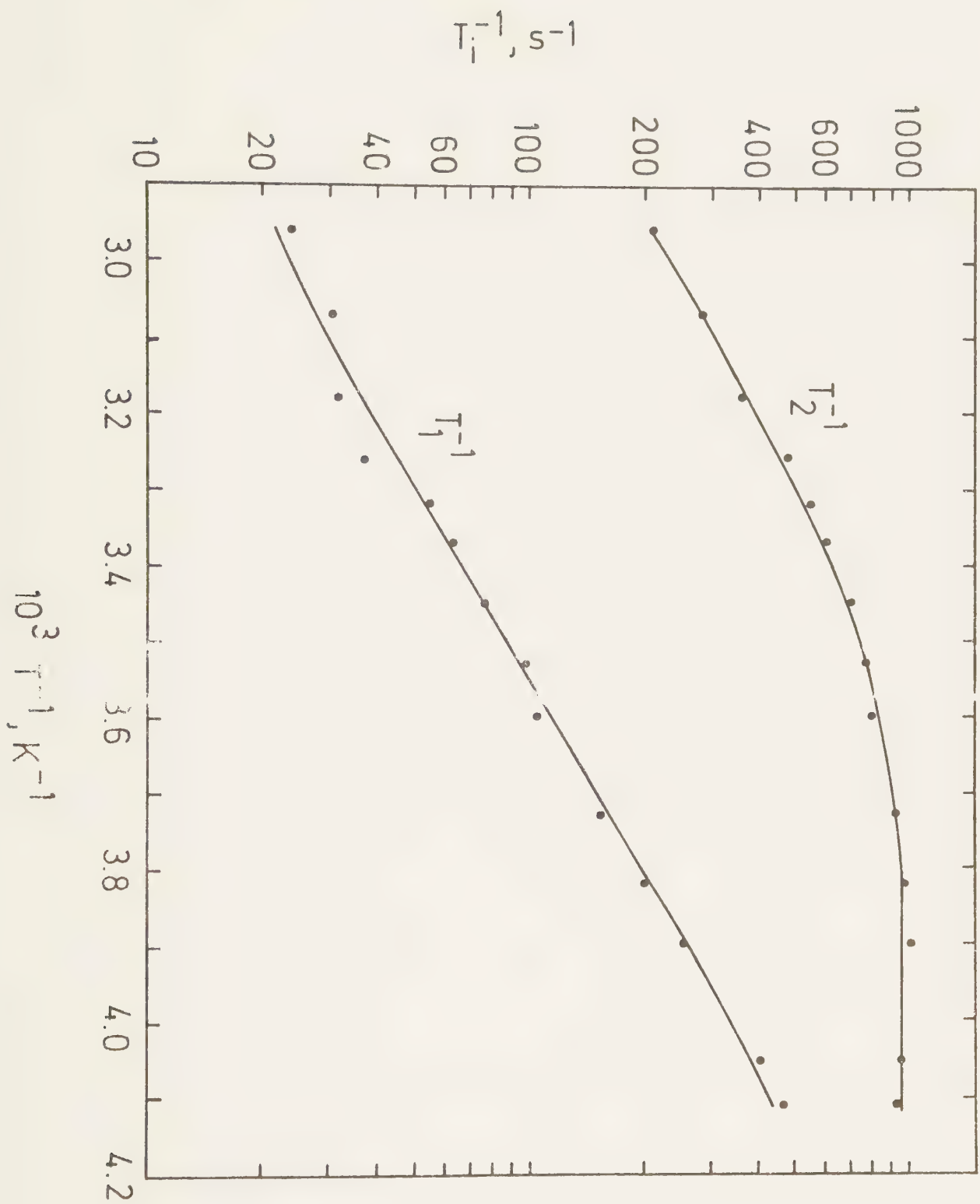
Captions to figures

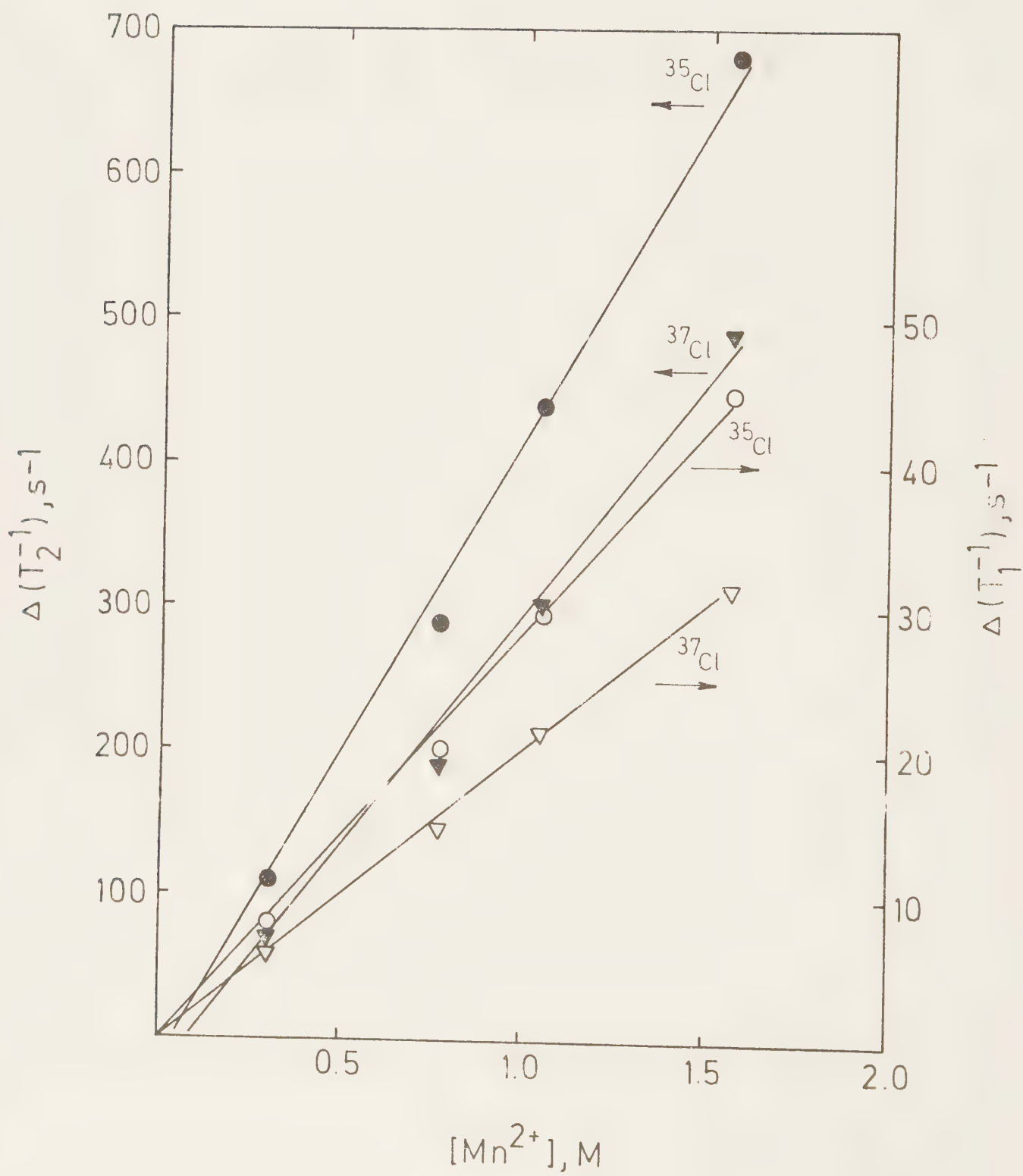
Figure 1. The water proton relaxation rates for a solution of Mn^{2+} at 61 MHz as a function of the inverse temperature. The solution contained 6.54 mM Mn^{2+} and 6.28 M NaClO_4 . The continuous curve was drawn with help of the best fit parameters in table 1.

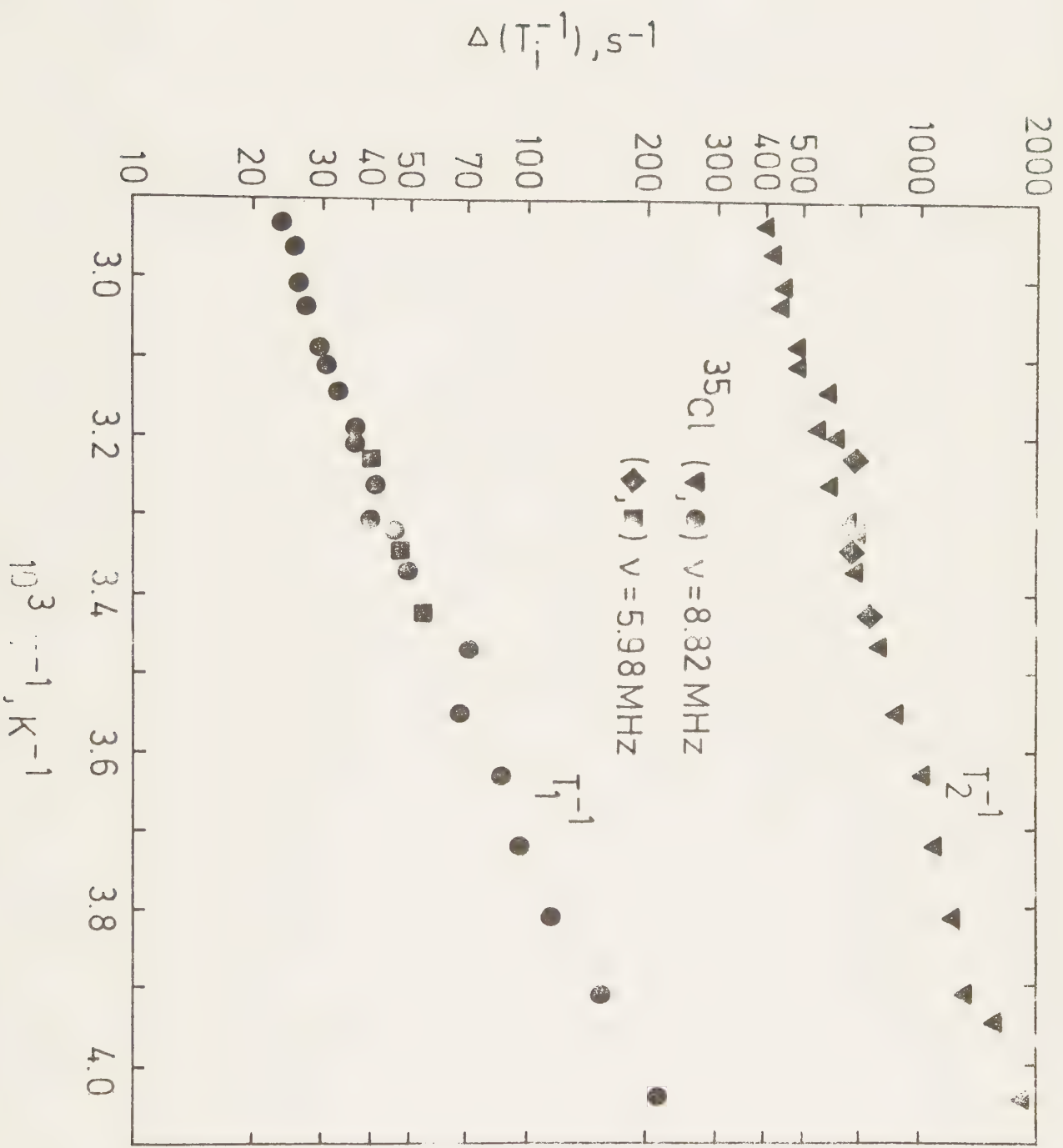
Figure 2. The excess ^{35}Cl and ^{37}Cl relaxation rates in a 6.28 M ClO_4^- solution as a function of manganese concentration with Na^+ as the other cation. The relaxation rates were obtained at 2.07 T ($\nu_{^{35}\text{Cl}} = 8.826$ MHz, $\nu_{^{37}\text{Cl}} = 7.346$ MHz) and 300 K
(●) $\Delta(1/T_2)$ for $^{35}\text{ClO}_4^-$, (▼) $\Delta(1/T_2)$ for $^{37}\text{ClO}_4^-$,
(○) $\Delta(1/T_1)$ for $^{35}\text{ClO}_4^-$ and (▽) $\Delta(1/T_1)$ for $^{37}\text{ClO}_4^-$.
The left-hand scale pertains to the transverse relaxation rates $\Delta(1/T_2)$ and the right-hand scale to the longitudinal relaxation rates $\Delta(1/T_1)$. The lines represent results of the least-squares fits.

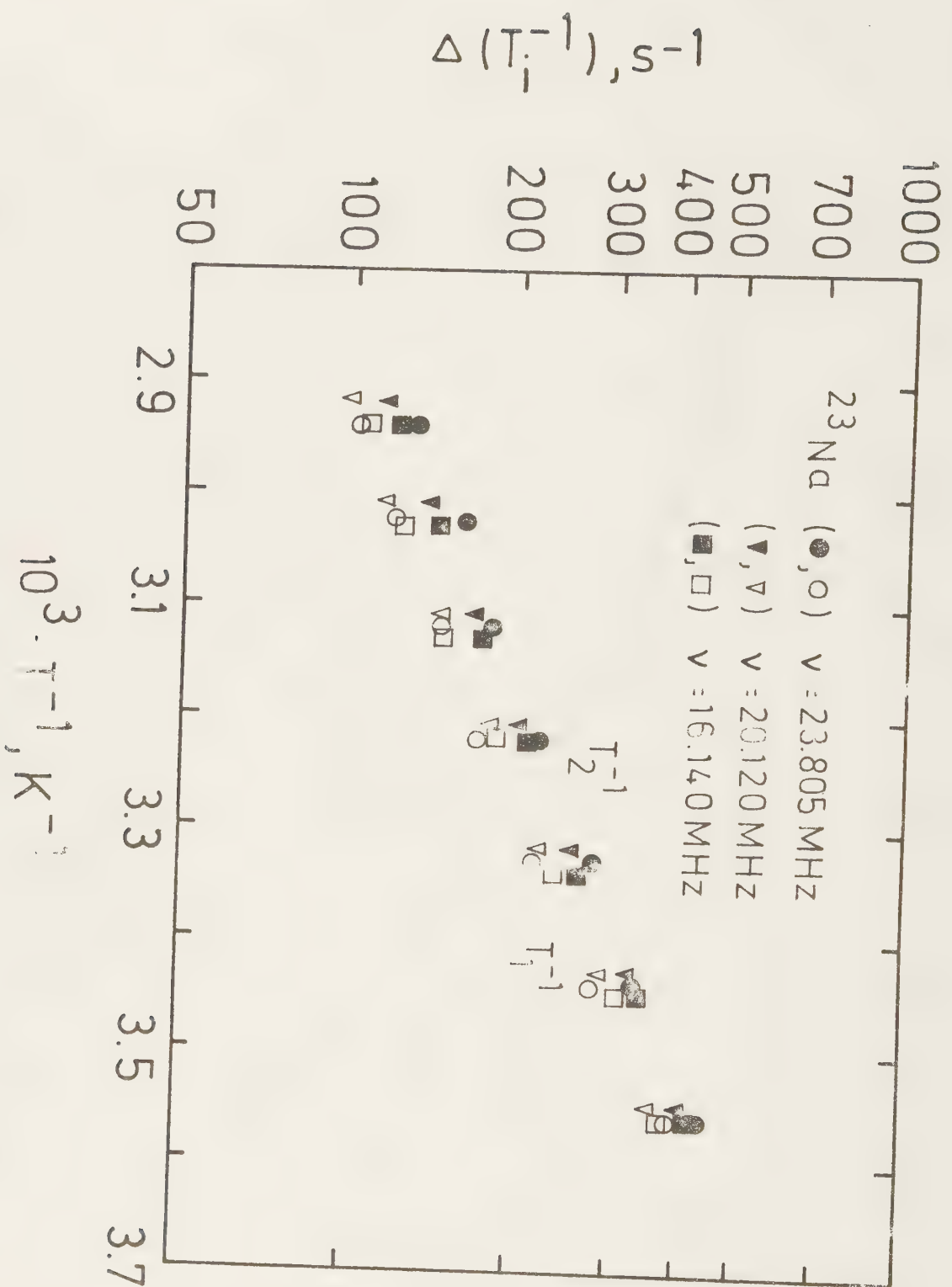
Figure 3. The temperature dependence of the excess $^{35}\text{ClO}_4^-$ relaxation rates in a solution composed of 6.28 M ClO_4^- , 3.14 M Na^+ and 1.57 M Mn^{2+} . (▼) $\Delta(1/T_2)$ at 8.82 MHz, (◆) $\Delta(1/T_2)$ at 5.98 MHz, (●) $\Delta(1/T_1)$ at 8.82 MHz and (■) $\Delta(1/T_1)$ at 5.98 MHz.

Figure 4. The temperature and frequency dependence of the excess $^{23}\text{Na}^+$ relaxation rates in a solution composed of 3.14 M Na^+ , 6.28 M ClO_4^- and 1.57 M Mn^{2+} . Filled symbols denote $\Delta(1/T_2)$ at 23.805 MHz (●), 20.120 MHz (▼) and 16.14 MHz (■). Open symbols refer to $\Delta(1/T_1)$ at 23.805 MHz (○), 20.120 MHz (▽) and 16.140 MHz (□).









IX

^{35}Cl NMR STUDIES OF ANION BINDING TO HALOPHILIC PROTEINS:
FERREDOXIN FROM HALOBACTERIUM OF THE DEAD SEA

Pétur Reimarsson, Björn Lindman and Moshe M. Werber*
Physical Chemistry 2, Chemical Center, P.O.B. 740,
S-220 07 Lund, Sweden and Polymer Department,
The Weizmann Institute of Science, Rehovot,
Israel.

*Present address: Department of Hematology, Hadassah
University Hospital, Kiryat-Hadassah, Jerusalem,
Israel.

1. Introduction

In recent years there has been great interest in the characteristics and properties of the extremely halophilic bacteria isolated from the Dead Sea and other saline environments [1] and several proteins from these bacteria have been purified. In general these proteins require high concentrations of salts for optimum stability as opposed to non-halophilic proteins which often are denatured and dissociated under similar conditions. An interesting question is therefore how the halophilic proteins are adapted to the high salt concentrations and what constitutes the molecular basis of the high salt stability. One characteristic feature of the halophilic proteins is that they carry a large excess of negative charges when compared to their non-halophilic counterparts [1-4]. It is therefore of interest to elucidate to what extent the halophilic proteins can bind anions, which are known to interact strongly with a large number of non-halophilic proteins. ^{35}Cl nmr has proved to be a valuable tool for studying the interactions of anions with proteins [5-6]. It has been found that anions interact nonspecifically with charged groups on the protein surface and that the forces governing this interaction are dispersion forces in combination with nonspecific electrostatic forces [7]. Here we report ^{35}Cl nmr studies of anion binding to 2Fe-ferredoxin(FD) isolated from Halobacterium of the Dead Sea. Specifically we have studied the effect of competing anions on the binding of Cl^- to FD and we have also obtained the binding constant for Cl^- binding to FD.

2. Experimental

FD was isolated from Halobacterium of the Dead Sea and purified as described [3]. The solutions were made from salts of analytical grade and filtered. FD concentration was determined from the absorbance at 420 nm and in all the solutions the ratio A_{420}/A_{280} was found to be 0.31 ± 0.01 . The ^{35}Cl nmr line-width studies were made at 9.80 MHz on a Varian XL-100-15 spectrometer operating in the Fourier transform mode. External lock was employed and the acquisition

time was 0.2 s. The number of transients varied from 500 to 4000 depending on solution and linewidth. Each reported linewidth is the average of at least three separate measurements. The linewidth is related to the transverse relaxation rate by $\pi \cdot \Delta\nu = R_2$ where $\Delta\nu$ is the linewidth and R_2 the relaxation rate.

3. Theory

^{35}Cl is a quadrupolar nucleus with spin quantum number 3/2. Its relaxation is mainly due to interactions between its quadrupole moment and fluctuating electric field gradients at the nucleus. Both the longitudinal and the transverse relaxation are in general described by two exponentials, but can be approximated by one exponential when $\omega\tau_c \leq 1.5$ (see below) [8]. The relaxation rate equations for the chloride exchanging between a site on a macromolecule and the solution are therefore [9]

$$R_1 = P_F R_0 + \frac{2\pi^2}{5} P_B \chi^2 \tau_c \left(\frac{0.2}{1+\omega^2 \tau_c^2} + \frac{0.8}{1+4\omega^2 \tau_c^2} \right)$$

$$R_2 = P_F R_0 + \frac{2\pi^2}{5} P_B \chi^2 \tau_c \left(0.6 + \frac{0.4}{1+\omega^2 \tau_c^2} + \frac{1}{1+4\omega^2 \tau_c^2} \right)$$

in the case of fast exchange which applies to all the cases reported here as shown from the temperature dependences. P_F and P_B are the fractions of chloride ions which are free and bound, respectively (here $P_F \approx 1$), R_0 is the relaxation rate in the absence of the macromolecule, χ is the quadrupole coupling constant which is a measure of the strength of interaction between the ion and the macromolecule, τ_c is the correlation time for the bound ions and ω is the resonance frequency (expressed in radians/s). The excess relaxation rates $\Delta R_{1,2} = R_{1,2} - R_0$ describe the relaxation of chloride bound to the macromolecule. The correlation time can be extracted from the ratio or the excess relaxation rates. In the case where the nmr absorption

signal is recorded, the lineshape can be fitted to two Lorentzians to give the correlation time [10].

4. Results and Discussion

Figure 1 shows the excess ^{35}Cl linewidth ($\Delta\nu_{\text{excess}} = \Delta\nu - \Delta\nu_0 = \frac{1}{\pi}(R_2 - R_0)$) for halophilic ferredoxin as a function of the inverse temperature. First we discuss the two curves representing two different chloride concentrations (1.8 and 4.3 M NaCl). The excess linewidth is proportional to the fraction of ions bound (P_B) and assuming that all the binding sites are similar one can write

$$P_B = \frac{n \text{ FD} \cdot K}{1 + K \cdot \text{Cl}^-}$$

where n is the number of binding sites, FD is the ferredoxin concentration and K is the binding constant of Cl^- to FD . The ratio of the excess linewidths at two different Cl^- concentrations thus gives a value for the binding constant. We obtain $K \approx 0.09 \text{ M}^{-1}$ at 28°C and this is ten times less than the binding constant for chloride binding to the weak sites of human serum albumin [7]. Furthermore, the binding constant decreases as the temperature is lowered. This corresponds to a distinctly positive value for the binding enthalpy. The information on the thermodynamics of Cl^- binding to proteins is rather sparse. It is interesting to note, however, that for human serum albumin there is a large negative binding enthalpy for the strong Cl^- binding sites while the enthalpy change is close to zero for the weak binding sites [12, 13]. A negative binding enthalpy for a strongly negative net charge is in line with this, but a rationalization is difficult since one has to take into account hydration enthalpies of the positively charged groups on the protein and of the anions as well as the enthalpy of the electrostatic ion-ion interaction. Because of the long-range nature of electrostatic interactions the latter can be

assumed to vary considerably with the protein net charge.

When the temperature is lowered, the molecular motion slows down and $\omega\tau_c$ becomes larger; it is then possible to fit the lineshape to two Lorentzians and obtain the correlation time [10]. For chloride ions bound to ferredoxin the value obtained is $\tau_c \approx 10$ ns (in 4.3 M NaCl at -7.0°C), which is close to the value expected for protein reorientation from the Debye-Stokes-Einstein relation, for a protein of this size (MW 14000). As we know the binding constant, then if the number of binding sites is known, the quadrupole coupling constant can be calculated. By taking the number of binding sites as 7 (3 Lys, 3 Arg, 1 His) [3] we obtain $\chi \approx 3.6$ MHz which is in close agreement with what is expected for Cl^- binding to a positively charged amino acid residue [8], but much less than what would be expected for the binding of Cl^- to a metal ion such as Fe^{3+} [5].

We now consider the effect of adding 2.5 M of competing anion to a 1.8 M NaCl-ferredoxin solution. The competing anions should expel chloride ions from the protein, thereby reducing the fraction of chloride bound and hence also the excess linewidth. The more pronounced the effect, the stronger is the binding of the competing ion. From figure 1 it is seen that the ions bind according to the sequence $\text{SCN}^- > \text{Cl}^- \approx \text{ClO}_4^-$, which deviates from the lyotropic series for the anions where perchlorate is found to bind more strongly than chloride [13]. From the figure it is also inferred that addition of sulfate greatly increases the linewidth. Since this corresponds to increased Cl^- binding some process other than simple competition must be taking place. As to the nature of this process we can only put forward some suggestions. Thus the sulfate may change the hydration state of the protein; on the other hand the hydration of the chloride ions may be affected or a combination of both of these effects could occur.

In any event it is striking that even a highly acidic protein such as halophilic ferredoxin, that contains about 40 negatively-charged and only 6-7 positively-charged residues [3], will still retain several anion binding sites, which is a characteristic feature

of most proteins.

Acknowledgements

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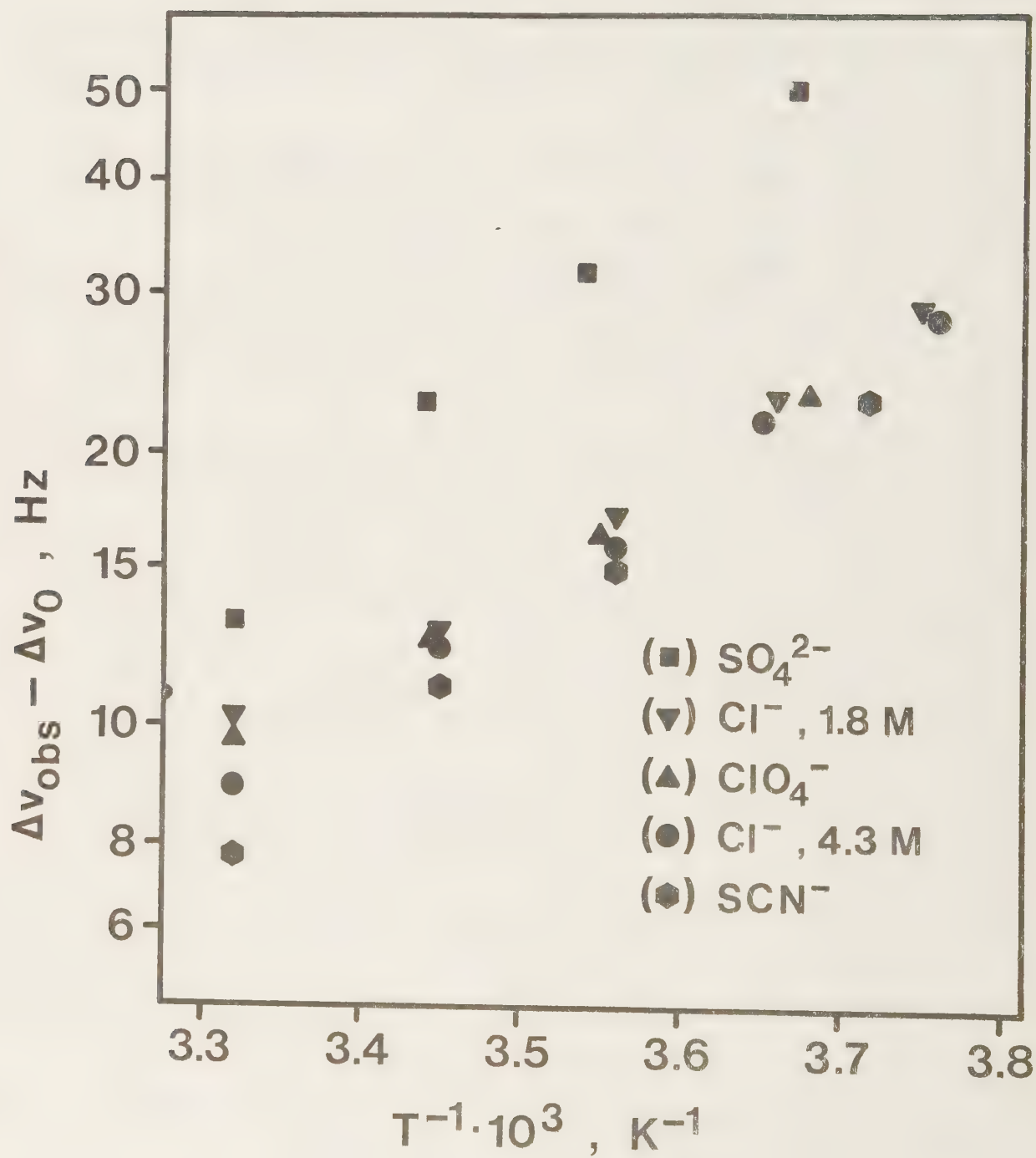
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Figure caption

Fig. 1. The excess ^{35}Cl linewidths for ferredoxin in various salt solutions as a function of the inverse temperature. The linewidths have been normalized to a ferredoxin concentration of 23.5 mg/ml. All solutions contain 10 mM phosphate buffer at pH 7.3

(●) 4.3 M NaCl. (▼) 1.8 M NaCl, (■) 2.5 M $(\text{NH}_4)_2\text{SO}_4$ and 1.8 M NaCl, (▲) 2.5 M NaClO_4 and 1.8 M NaCl, (⬢) 2.5 M NaSCN and 1.8 M NaCl.



XI

DNA AS A POLYELECTROLYTE:
MONOVALENT CATION BINDING BY ^{23}Na NMR

by

Dennis R. Burton, Sture Forsén*, Hans Gustavsson and
Pétur Reimarsson

Department of Physical Chemistry 2,
Chemical Centre, University of Lund,
P.O.Box 740,
S-220 07 LUND
Sweden

*To whom correspondance should be addressed.

One of the most important factors that determine the physico-chemical and biological properties of DNA (deoxyribonucleic acid) is the high electrostatic charge due to ionised phosphate groups.

Recently the ion binding properties of DNA have been thoroughly discussed by Manning (1). One of the main points raised by Manning is that the ion binding cannot in a simple way be described in terms of equilibrium constants, while it is possible to rationalise a large body of experimental data within a simple electrostatic model. For example, the concept of an ion condensation that occurs above a certain critical linear charge density (1e per 72 Å) of the macromolecule gives a good first order description of the ion binding properties.

Nuclear magnetic resonance (NMR) studies of ion binding to macromolecules is an established method for studies of the binding properties on a molecular level (2). Thus the relaxation rates of bound ions such as $^{23}\text{Na}^+$ are markedly enhanced and therefore the $^{23}\text{Na}^+$ relaxation behaviour can, under suitable conditions be analysed to yield quantitative information on the environment and motional characteristics of the Na^+ ion in interaction with DNA. These should be consistent with our view of DNA as a polyelectrolyte and this aspect is investigated in this communication.

$^{23}\text{Na}^+$ is a quadrupole nucleus with spin quantum number $I = 3/2$. Its relaxation is mainly due to interactions between its quadrupole moment and time-varying electric field gradients at the nucleus. The electric field gradients are related to the electronic configuration around the nucleus and their coupling to the nuclear quadrupole moment expressed in a nuclear quadrupole coupling constant (χ). The time variation of the field gradients depends on the motional properties of Na^+ and is expressed in a correlation time (τ_c). Finally Na^+ bound to e.g. DNA is not observed directly - rather an averaging between free and bound sites generally takes place (fast-exchange). Thus $^{23}\text{Na}^+$ relaxation in the bound state is a function of χ , τ_c and the fraction of bound ions, p_B .

Relaxation is also dependent on the magnitude of the nuclear Larmor frequency, ω , relative to τ_c^{-1} . If $\omega\tau_c \ll 1$ (extreme narrowing), the resonance line shape is Lorentzian, the longitudinal (T_1) and transverse (T_2) relaxation times are equal and both are frequency independent and proportional to the product $p_B\chi^2\tau_c$ (2). Relaxation measurements can then only yield one of these three parameters if the other two are known. As $\omega\tau_c$ approaches and becomes greater than unity (non-extreme narrowing) the resonance line shape becomes a superposition of two Lorentzians corresponding to a biexponential decay of the transverse magnetisation, the longitudinal magnetisation behaving in a similar way. When $\omega\tau_c \leq 1.5$ linearised expressions yielding effective T_1 's and T_2 's may be derived (3). A number of methods for the determination of τ_c and the product $p_B\chi^2$ then become available according to the magnitude of $\omega\tau_c$ (3).

For Na^+ interacting with DNA, the magnitude of τ_c is such that only at the high field (large ω) of a superconducting magnet are non-extreme narrowing conditions clearly apparent. For a solution of DNA and Na^+ , analysis shows a deviation of the ^{23}Na resonance from a Lorentzian line-shape on increasing the magnetic field from 2.3T to 6T. Even more striking is the non-Lorentzian line-shape of the ^{23}Na resonance for Na^+ interacting with single stranded DNA at either magnetic field which can clearly be seen in Fig. 1. This deviation is marked by the fact that the resonance line becomes broader near the baseline and narrower near the top. Table 1 summarises the τ_c values obtained by various methods.

τ_c for both calf thymus and E. coli DNA is found to be 1.8 ± 0.3 ns at 4°C and 1.1 ± 0.3 ns at 23°C . This short correlation time is consistent with either rapid exchange of Na^+ ions from the bulk solution to the DNA surface or a rapid diffusion along the same surface. Reuben *et al.* (4) found a τ_c faster than 5.5 ns. τ_c for single-stranded DNA is considerably longer and consistent with a slower exchange from the more isolated negative charges of the single-stranded species (where the gradient of the potential is larger) or a slower

diffusion along the single-stranded species because of the greater spacing between charges.

To obtain the quadrupole coupling constant, χ , for the Na^+ -DNA interaction from the ^{23}Na relaxation data it is necessary to assume a value for the fraction of ions "bound", p_B . The number of monovalent cations within a given distance from the DNA cylinder can be obtained by a numerical solution of the Poisson-Boltzmann equation when DNA is assumed to be uniformly charged. It is thus found that roughly 40% of the charges on DNA are neutralised by sodium ions within 3Å from the cylinder (5). From this we obtain χ to be 260 ± 30 kHz. According to Mannings ion condensation model 76% of the DNA-phosphates are expected to be neutralised by Na^+ ions (1,6) and this gives χ to be 180 ± 20 kHz. Both values are considerably less than that observed for free aqueous Na^+ (approx. 1 MHz) and this is consistent with findings for other polyanions (2) and what has previously been found for the Na^+ /DNA interactions (4). Given this χ it seems highly improbable that Na^+ loses any water of hydration in its interaction with DNA which is indeed in agreement with the short τ_C . For Na^+ bound to single stranded DNA χ is found to be 250-300 kHz assuming a p_B used by Manning (1,6) (linear charge density parameter = 1.8). If single stranded DNA is assumed fully extended χ is obtained as 750-900 kHz.

We have further investigated, in a similar way, the interaction of Na^+ and transfer RNA (tRNA), which can also be regarded as a polyelectrolyte. τ_C is found to be roughly twice as long as for DNA (Table 1). At present we have no readily applicable model from which to estimate p_B for tRNA but use of reasonable values shows that χ is similar to that for DNA. We thus conclude that the interaction of Na^+ with tRNA is similar to that with DNA although differences do appear in the finer details.

We are indebted to H. Wennerström for helpful discussions. This work was supported by the Swedish Natural Science Research Council.

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Table 1

The correlation times of Na^+ bound to double helical and single stranded DNA as obtained by various methods according to (2). Also shown is a value for tRNA.

The DNA concentrations used varied between 3mM and 17mM in phosphate and sodium concentrations between 7mM and 62mM. All solutions contained sodium phosphate buffer at pH 6.8.

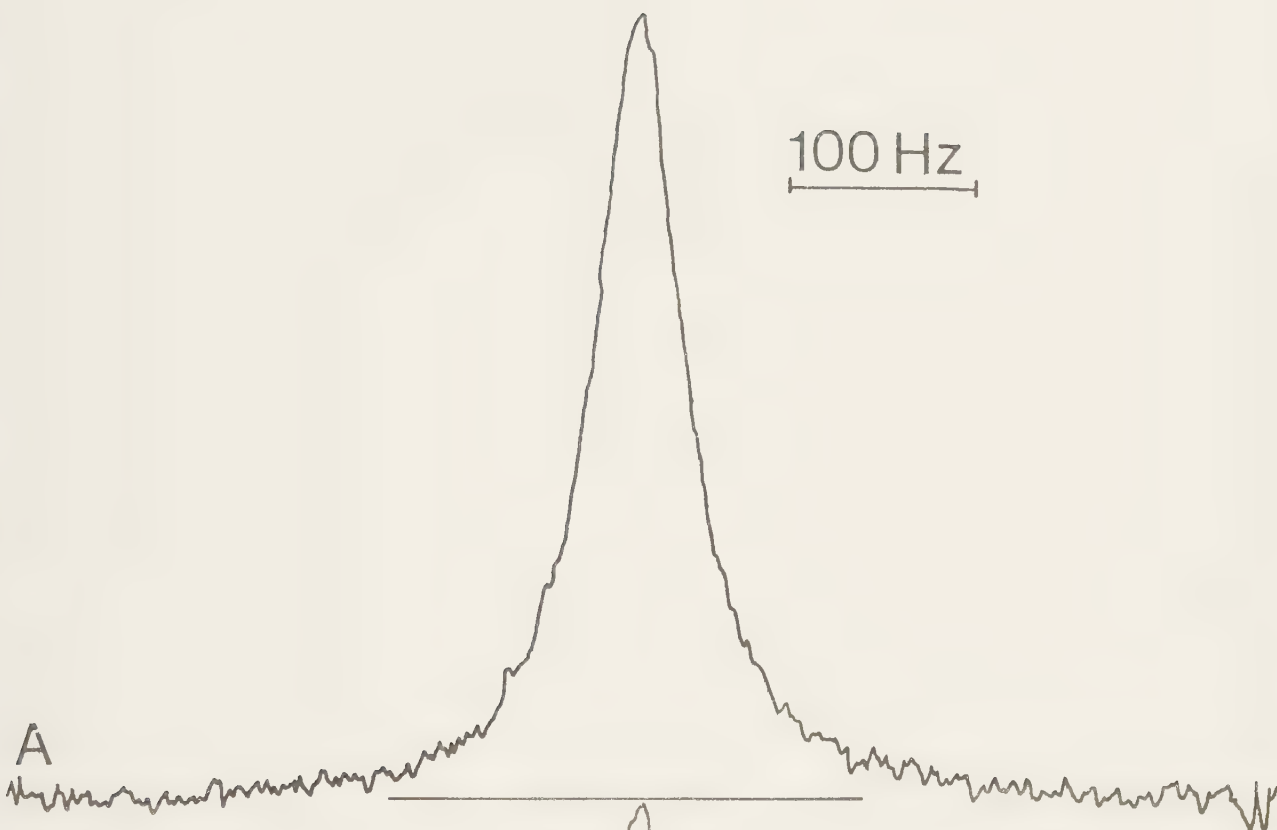
$\tau_{c,ns}$	from T_1/T_2	4°C from lineshape analysis	from frequency depen- dence of T_1	23°C T_1/T_2
Calf thymus DNA double-helical	1.9	1.8	1.5	1.2
E. Coli DNA double-helical	1.8	1.7	-	1.0
Calf thymus DNA single-stranded	-	9.0	-	-
E. Coli DNA single-stranded	-	8.0	-	-
transfer RNA (unspecified)	-	-	-	2.1

Caption to figure

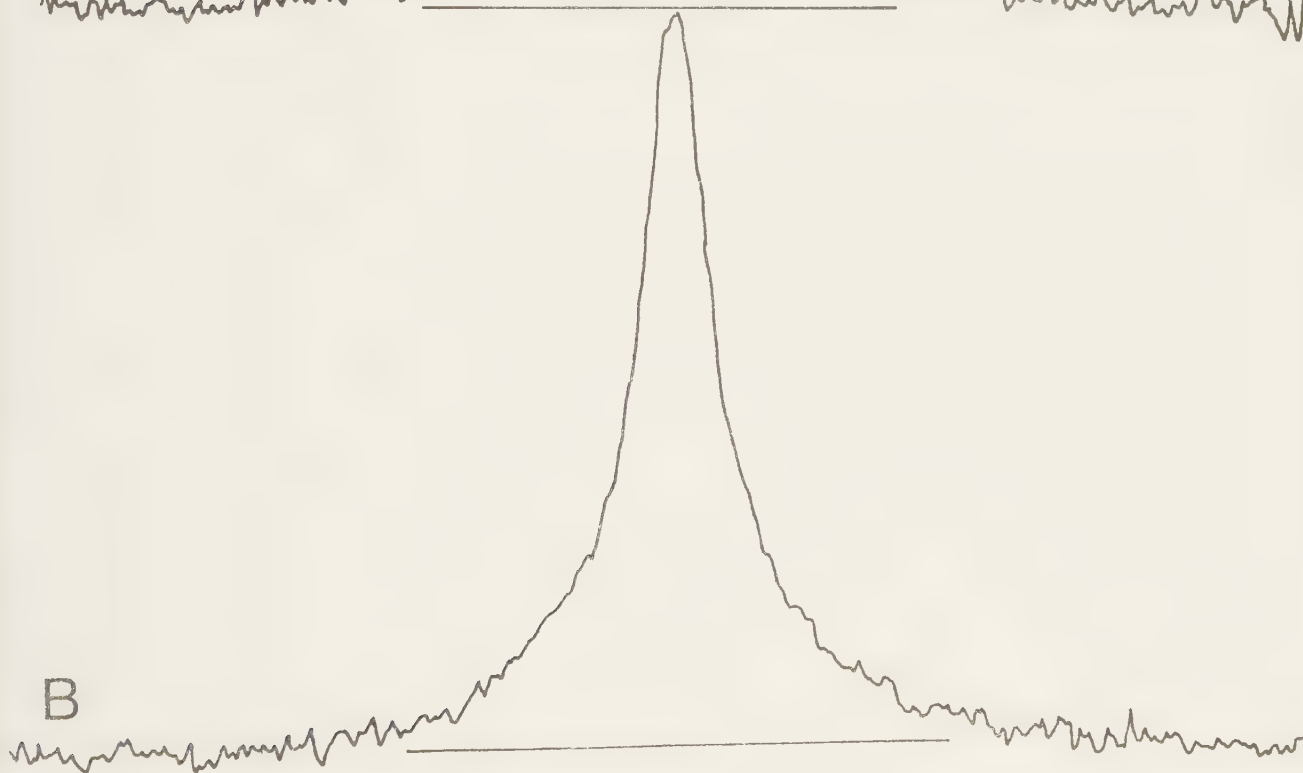
Figure 1. ^{23}Na NMR signal at 2.3 T of (A) double-helical and (B) single stranded E. Coli DNA. The concentration of DNA is 7mM in phosphate and the total sodium concentration is 16mM. The solution is buffered at pH 6.8 with sodium phosphate. The temperature is 4°C.

100 Hz

A



B



XII

COUNTERION NMR IN POLYELECTROLYTE SOLUTIONS.

PRELIMINARY STUDY OF $^{25}\text{Mg}^{2+}$ and $^{43}\text{Ca}^{2+}$ INTERACTION WITH DNA.

Pétur Reimarsson¹, Joseph Parello², Torbjörn Drakenberg¹,
Hans Gustavsson and Björn Lindman¹.

¹ Physical Chemistry 1 and 2, Chemical Center, P.O.B. 740,
S-220 07 LUND, Sweden

² Equipe de Recherche de Biophysique No. 140, CNRS USTL,
340 60 MONTPELLIER, France.

Introduction

The importance of electrostatic effects on the physico-chemical and biological properties of nucleic acids has long been recognised. It is found that ion binding to nucleic acids cannot in a simple way be described in terms of equilibrium constants; therefore there has been a rapidly increasing interest in polyelectrolyte systems concerning mainly the development of models amenable to theoretical analysis and the implications of polyelectrolyte phenomena in biological situations [1-5]. The concept of counterion condensation advocated by Manning (for a review see [1]) has been found to give a good first order description of the interaction of ions with polyelectrolytes. According to this model the condensation occurs above a certain critical linear charge density (1 elementary charge per 7.1 Å for monovalent counterions and one charge per 14.2 Å for divalent counterions) and in a solution contains only one type of counterion the charge fraction of the polyelectrolyte remains constant over a broad concentration range.

For the study of counterion/polyion interactions at a molecular level, NMR should be a most useful method, and previously it has been shown, with $^{23}\text{Na}^+$ and $^{35}\text{Cl}^-$ as examples, that quadrupolar effects in NMR give significant information on the mode and dynamics of ion binding [6-9].

In particular the motional characteristics of Na^+ bound to DNA and tRNA have recently been elucidated [9]. From a biological point of view, the study of Mg^{2+} and Ca^{2+} is especially intriguing. However, as can be learnt from a recent comprehensive review [10], available isotopes have unfavourable NMR characteristics and, therefore, Mg and Ca are among the least studied elements. The use of isotope-enriched ^{25}Mg and ^{43}Ca considerably improves the situation and it was recently found that the interactions of Mg^{2+} [11] and Ca^{2+} [12] with a muscular protein, parvalbumin, produce marked effects in ^{25}Mg and ^{43}Ca quadrupole relaxation. However, one problem was encountered, i.e. for high affinity binding to proteins, chemical exchange is often too slow to allow NMR studies.

Also $^{25}\text{Mg}^{2+}$ NMR has been used to study binding properties of bovine serum albumin and nitrogenase Fe protein [13]. Robertson et al. have shown that the quadrupole relaxation for $^{25}\text{Mg}^{2+}$ is sufficiently effective to give large line broadening effects also when the metal is bound to smaller ligands, like small peptides, whereas for $^{43}\text{Ca}^{2+}$ the quadrupole relaxation is not so effective and in this case chemical shifts due to complexation could be observed [14,15].

It was anticipated in ref. 12 that the Ca^{2+} and Mg^{2+} ion exchange should be faster when bound to biological poly-electrolytes than to specific sites in proteins, and as will be shown in this preliminary report, with DNA as an example, the metal ion binding may indeed be profitably explored.

Experimental

Calf thymus DNA of low molecular weight (about 3.6×10^5 daltons; $S_{20} = 7.7$) was prepared as previously described [16]. The DNA-phosphate concentration was determined by the absorbance at 260 nm assuming that 1.0 OD^{260} is equivalent to $1.5 \times 10^{-4} \text{ M DNA-P}$. ^{43}Ca and ^{25}Mg were purchased from Oak Ridge, Tennessee, USA, as $^{43}\text{CaCO}_3$ (61% enrichment) and ^{25}MgO (98% enrichment), respectively. The ^{25}Mg and ^{43}Ca line-width studies were made at 6.095 MHz and 6.73 MHz respectively on a Varian XL-100-15 spectrometer operating in the FT mode. External protein lock was employed, the acquisition times were 0.1 s for ^{25}Mg and 0.2 s for ^{43}Ca . The number of transients varied from 500 to 10 000 depending on line-width and solution. Each reported line-width is the average of at least two different measurements and the resulting errors are less than 5%. Temperature was maintained by a stream of dry thermostated gas and is accurate to 0.5°C .

Results and discussion

In Figs. 1 and 2 we report the temperature dependence of $^{25}\text{Mg}^{2+}$ and $^{43}\text{Ca}^{2+}$ relaxation, respectively, in the presence of DNA. (In the absence of DNA, relaxation is much smaller and decreases monotonously with increasing temperature, cf. for example ref. 11). The experimental results

are given as line-widths which are related to the transverse relaxation time, T_2 , as $T_2^{-1} = \pi \Delta \nu_{\frac{1}{2}}$. The important features of Fig. 1 are the marked increase in relaxation rate with increasing temperature and the low relaxation rate above the DNA melting point which is considered to correspond to a transition from a double-stranded helical DNA to a single-stranded random coil state. For $^{25}\text{Mg}^{2+}$ (Fig. 2) there is likewise a marked decrease in relaxation at DNA melting while below this temperature there is a slow variation with a wide maximum. A similar curve is obtained for transfer RNA [17]. Parallel optical studies of the denaturation of double-stranded DNA in the presence of Ca^{2+} and Mg^{2+} show that the melting transition occurs at 80-85°C under conditions similar to those used for the NMR experiments.

The principles of ion quadrupole relaxation studies of macromolecular systems have recently been reviewed [18,19]. (In ref. 18 a detailed account of the theory is given.) For both ^{25}Mg and ^{43}Ca , quadrupole interactions give the dominating relaxation mechanism. The observed relaxation can be written as a sum of contributions from different sites, i.e.

$$\pi \Delta \nu_{\frac{1}{2}} = T_2^{-1} = \sum p_i / (T_{2i} + \tau_{\text{ex},i})$$

Here p_i is the fraction of ions in site i , $\tau_{ex,i}$ is the life-time of the ion in site i and T_{2i} is the intrinsic relaxation time of site i . $T_{2i}^{-1} = K\chi^2 \tau_c$ where $K = 0.947$ for ^{25}Mg (spin 5/2) and 0.403 for ^{43}Ca (spin 7/2), χ is the quadrupole coupling constant describing the strength of the interaction between the ion and the site and τ_c the correlation time. Here we have assumed that the extreme-narrowing condition applies i.e. $\omega\tau_c < 1$ (ω is the resonance Larmor frequency in rad/s) which is not established for these ions and does not affect the general conclusions of this work.

Current polyelectrolyte theories [1-5] relate counter-ion binding to the charge density of the cylinder or to a linear charge density; the ion condensation model of Manning [1] is in qualitative agreement with solutions of the Poisson-Boltzmann equation for different geometries [20]. The linear charge density is extensively decreased at the melting of DNA and the dramatic counterion relaxation changes at high temperatures are in qualitative agreement with the transition from a double- to a single-stranded DNA. According to the Manning ion condensation model [1], considering only the linear charge density, the Mg^{2+} and Ca^{2+} binding should change from ca 88% polyion charge neutralization to a much lower level (ca 50%). From the temperature dependence of counterion relaxation it can be deduced, at least for Ca^{2+} , that $\tau_{ex,i}$ dominates over T_{2i} 18 . With the aid of Manning's condensation model (88%

charge neutralisation) and the measured line broadenings the average lifetime of Mg^{2+} bound to DNA was estimated to be ca. 0.4 ms at 4°C while Ca^{2+} has an average lifetime of ca. 9 ms at 25°C and ca. 3 ms at 50°C . It is clear that analysis of the data of Figs. 1 and 2 in terms of activation parameters must be preceded by rather detailed studies of the binding equilibria.

If the ion condensation model were applicable under the conditions of the present work, one would expect the difference between the observed line width and that of a solution without DNA to be proportional to the DNA concentration at a constant ion concentration. As is shown in Fig. 3, there is a marked non-linearity in the plot of line width of ^{25}Mg versus DNA concentration which is in contrast to many other macromolecular systems [18]. It has also been observed that the excess line-width of $^{23}\text{Na}^{+}$ in transfer RNA solutions is not linear with respect to tRNA concentration [17]. However, it is generally not expected that the charge fraction of polyelectrolyte is independent of concentration.

The present preliminary report clearly demonstrates the feasibility of NMR studies of Ca^{2+} and Mg^{2+} interaction

with DNA and also shows that one has direct access to both kinetics and thermodynamics of ion binding. The kinetic information is particularly difficult to obtain by other techniques and the marked difference in exchange kinetics between Mg^{2+} and Ca^{2+} , which probably reflects a difference in hydration state of the bound ions, will be examined in detail.

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Figure 1. Study of the Ca^{2+} -DNA system by ^{43}Ca NMR.

The dependence of the linewidth on temperature for a solution containing 7.1 mM DNA-P, 35 mM Ca^{2+} , 57 mM Na^+ in 13 mM Tris-HCl at pH 5.2.

Insert: Effect of DNA concentration on the ^{43}Ca linewidth. Successive volumes of a stock DNA solution (4.25×10^{-2} M DNA-P in 75 mM Tris-HCl at pH 7.1) added to a 1.53 ml solution containing 40 mM Ca^{2+} , 65 mM Na^+ in 15 mM Tris-HCl at pH 7.1. Temperature 27°C . Total volume variation 13%.

Figure 2. Dependence of the ^{25}Mg linewidth on temperature for a solution containing 7.1 mM DNA-P, 88 mM Mg^{2+} , 21 mM Na^+ in 50 mM Tris-HCl at pH 7.4.

Figure 3. Dependence of the ^{25}Mg excess linewidth on DNA concentration at 28°C . Successive volumes of a stock DNA solution (1.54×10^{-2} M in 75 mM Tris-HCl at pH 7.1) added to a 1.50 ml solution containing 96 mM Mg^{2+} , 17 mM Na^+ in 15 mM Tris-HCl at pH 7.1. Total volume variation 13%.

